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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In the Application of:	)	
	)	Atty. Docket No. 00-1278
Lawton *et al.*	)	
	)	Art Unit: 1645
Serial No.: **09/765,739**	)	
	)	Examiner: V. Ford
Filed: **January 18, 2001**	)	
	)	Conf. No.:

For: **Compositions and Methods for Detection of *Ehrlichia canis* and *Ehrlichia chaffeensis* Antibodies**

BRIEF ON APPEAL

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For: **Compositions and Methods for Detection of *Ehrlichia canis* and *Ehrlichia chaffeensis* Antibodies**

BRIEF ON APPEAL

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Dear Sir:

An original and three copies of this appeal brief are submitted along with the large entity fee of three hundred dollars ($330) for filing an appeal brief. In the event of any variance between any of the amounts enclosed and the Patent and Trademark Office charges, the Commissioner is authorized to charge or credit any difference to our Deposit Account No. 13-2490.

Contents

REAL PARTY IN INTEREST

The real party in interest is IDEXX Laboratories, Inc., Westbrook, Maine, to whom this invention is assigned.

RELATED APPEALS AND INTERFERENCES

Applicant is aware of no related appeals or interferences concerning this application.

STATUS OF CLAIMS

Claims 21-24 and 39-42 are pending and stand rejected. Claims 1-20 and 25-34 have been withdrawn. Claims 35-38 have been cancelled. A copy of the claims is attached in Appendix A.

STATUS OF AMENDMENTS

An amendment after final was presented on February 17, 2004. Claims 39 and 41 were amended. The amendment was entered according to the Advisory Action issued on May 26, 2004. No further amendments were presented after final.

SUMMARY OF THE INVENTION

The invention provides a device containing one or more polypeptides consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and amino acid substitution variants thereof that specifically bind to an anti-*Ehrlichia* antibody. *See e.g.*, page 4, lines 1-5; page 5, lines 11-13; page 7, lines 14-17; page 8, line 21 through page 9, line 1; page 9, lines 8-11; page 15, lines 19-22; Table I. The device can comprise instructions for use of the one or more polypeptides for the identification of an *Ehrlichia* infection in a mammal. *See e.g.*, page 4, lines 1-5; page 15, lines 19-22. The

instructions for use can indicate that the identification of an *Ehrlichia* infection is done using a method of detecting presence of antibodies to *Ehrlichia* comprising:

(a) contacting one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and amino acid substitution variants thereof that specifically bind to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form;

(b) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present. *See e.g.,* page 3, lines 16-23; page 5, lines 11-13; page 7, lines 14-17; page 8, line 21 through page 9, line 1; page 9, lines 8-11; page 12, lines 5-16; Table 1.

The *Ehrlichia* infection can be caused by *Ehrlichia canis* or *Ehrlichia chaffeensis*. See e.g., page 11, lines 22-23.

The invention also provides a device containing one or more polypeptides selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 that specifically bind to an anti-*Ehrlichia* antibody. *See e.g.*, page 4, lines 1-5; page 5, lines 11-13; page 7, lines 14-17; page 8, line 21 through page 9, line 1; page 9, lines 8-11; page 15, lines 19-22; Table I. The device can further comprise instructions for use of the one or more polypeptides for the identification of an *Ehrlichia* infection in a mammal. *See e.g.,* page 4, lines 1-5; page 15, lines 19-22. The instructions for use indicate that the identification of an *Ehrlichia*

infection can be done using a method of detecting presence of antibodies to *Ehrlichia* comprising:

(a) contacting one or more polypeptides selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, that specifically bind to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form;

(b) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present. *See e.g.,* page 3, lines 16-23; page 5, lines 11-13; page 7, lines 14-17; page 8, line 21 through page 9, line 1; page 9, lines 8-11; page 12, lines 5-16; Table 1.

The *Ehrlichia* infection can be caused by *Ehrlichia canis* or *Ehrlichia chaffeensis.* See e.g., page 11, lines 22-23.

ISSUES

The issues presented to the Board for review by this appeal are:

I. Whether claim 21 is unpatentable under 35 U.S.C. §112, second paragraph as indefinite;

II. Whether claims 39-42 are unpatentable under 35 U.S.C. §102(a), as anticipated by Waner *et al.* J. Vet. Diagn. Invest., 2000, 12:240-244 ("Waner");

III. Whether claims 21-24 and 39-42 are unpatentable under 35 U.S.C. 102(a) as anticipated by Cadman *et al.,* Vet. Record, 1994, 135:362 ("Cadman"); and

IV. Whether claims 21-24 are unpatentable under 35 U.S.C. 102(a) as anticipated by Rikihisa, WO99/13720.

GROUPING OF THE CLAIMS

Claim 21 stands or falls alone. See Section I below.

Claims 39-42 stand or fall together. See Sections II and III below.

Claims 21-24 stand or fall together. See Sections III and IV below.

ARGUMENT

I. Claim 21 is definite under 35 U.S.C. §112, second paragraph.

Claim 21 stands rejected under 35 U.S.C. §112, second paragraph as allegedly indefinite. Applicants respectfully traverse the rejection.

The Office Action asserts that claim 21 is indefinite because it recites "amino acid substitution variants." The Office Action asserts that the specification does not define or disclose a structure for "amino acid substitution variants" and concludes that one of skill in the art would not know the structure of "amino acid variants." The test for definiteness is whether "those skilled in the art would understand what is claimed when the claim is read in light of the specification." *Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 1 USPQ2d 1081, 1088 (Fed. Cir. 1986). "If the claims, read in the light of the specification, reasonably apprise those skilled in the art both of the utilization and scope of the invention, and if the language is as precise as the subject matter permits, the courts can demand no more." *Shatterproof Glass Corp. v. Libbey-Owens Ford Co.*, 225 USPQ 634, 641 (Fed. Cir.), (quoting *Georgia-Pacific Corp. v. United States Plywood Corp.*, 118 USPQ 122, 132 (2d Cir.).

One of skill in the art would understand the meaning of amino acid substitution variants given the specification. The specification teaches amino acid substitution variants and how to identify and test such variants at, *inter alia*, page 7, line 14 through page 9, line 7. Additionally, a structural description of the claimed variants is provided by the specification. The variants are amino acid substitution variants that have at least 85% identity to SEQ ID NOs:1 through 7, and specifically bind to an anti-*Ehrlichia*

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antibody. Since the claimed amino acids are about 18 to about 20 amino acids long, an amino acid substitution variant has only about 3 amino acid substitutions at the most. The specification also discloses 7 related sequences that are useful in the invention. See specification, page 6. These 7 sequences can be considered variants of each other. When the sequences of SEQ ID NOs:1 through 7 are compared, highly conserved amino acids and partially conserved amino acids are revealed. See Table 1, Appendix B. Table 1 demonstrates that positions 3, 8, and 13 of SEQ ID NOs:1 through 7 are highly conserved across the seven sequences (see Table 1, dark gray columns).

Additionally, positions 1, 4, 5, 6, 7, 9, 10, 11, 12, and 15 are partially conserved across the 7 sequences (see Table 1, light gray columns). That is, only 2 different amino acids appear in these positions. For instance, only K or N appear as amino acids in position 1 across the seven sequences (see Table 1). One of skill in the art would recognize that the claimed variants should likely retain the amino acids at positions 3, 8, and 13 and that one of two amino acids should likely be present at positions 1, 4, 5, 6, 7, 9, 10, 11, 12, and 15. One of skill in the art would also recognize that amino acids at positions 2, 14, and 16-20 could tolerate a greater range of amino acid substitutions. Therefore, the specification provides structural guidance as to which amino acids can be changed so that the amino acid substitution variant polypeptides retain their biological function.

Therefore, one of skill in the art would understand what is claimed when the claim is read in light of the specification. Applicants respectfully request withdrawal of the rejection.

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II. Claims 39-42 are not anticipated by Waner under 35 U.S.C. §102(a).

Claims 39-42 stand rejected as under 35 U.S.C. §102(a) as anticipated by Waner. Applicants respectfully traverse the rejection.

The Office Action asserts that Waner teaches that use of a device comprising an *Ehrlichia* antigen. The Office Action incorrectly asserts that the claimed invention encompasses variants of SEQ ID NOs:1-7. The Office Action concludes that one of skill in the art could reasonably conclude that the *Ehrlichia* polypeptides taught by Waner are amino acid substitution variants of SEQ ID NOs:1-7 since the Applicant has provide no side-by-side comparison to show that the claimed polypeptides differ from the *Ehrlichia* polypeptides disclosed by Waner. The Office Action additionally asserts one of skill in the art could reasonably conclude that the "polypeptides" taught by Waner are substitution variants of the claimed polypeptides since the Waner "polypeptides" are used on a device (an immunocomb) to detect antibodies to *E. canis*.

Under 35 U.S.C. § 102, a claim is anticipated only if each and every element as set forth in the claim is found in a single art reference. *Verdegaal Bros. v. Union Oil Co.*, 2 USPQ2d 1051, 10533 (Fed. Cir. 1987); *In re Recombinant DNA Technology Patent and Contract Litigation*, 30 USPQ2d 1881, 1885 (S.D. Ind.1993) ("A patent is anticipated only if all the elements and limitations of the claims are found within a single, prior art reference."); *Structural Rubber Products Co. v. Park Rubber Co.*, 223 USPQ 1264, 1270 (Fed. Cir. 1984) (All elements of the claimed invention must be contained in a single prior art disclosure and must be arranged in the prior art disclosure as in the claimed invention); M.P.E.P § 2131. Furthermore, no difference may exist between the claimed

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invention and the reference disclosure, as viewed by a person of ordinary skill in the field of invention. *In re Recombinant DNA Technology Patent and Contract Litigation*, 30 USPQ2d 1881, 1885 (S.D. Ind.1993). Also, the identical invention must be described or shown in as complete detail as is contained in the claim. *Richardson v. Suzuki Motor Co.*, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989); *Chester v. Miller*, 15 USPQ2d 1333 (Fed. Cir. 1990); M.P.E.P. § 2131.

The claims recite a device containing one or more polypeptides selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, that specifically bind to an anti-*Ehrlichia* antibody.

Initially, it should be noted that Waner teaches whole *E. c*anis proteins and cells and does not teach or suggest the use of <u>any types</u> of *E. chaffeensis* polypeptides in a device. SEQ ID NOs:3-7 of the present invention are *E. chaffeensis*-derived polypeptides (see Table 1 of specification) and therefore cannot be anticipated by Waner.

Additionally, Waner does not teach or suggest the use of <u>distinct</u> polypeptides as shown in SEQ ID NOs:3-7. That is, Waner does not teach or suggest about 18 to about 20 amino acid polypeptides of SEQ ID NOs:3-7. Instead, Waner teaches an IFA for *E. canis* that uses DH82 cells that are heavily infected with *E. canis* as an antigen. See page 240, second column, last paragraph. Waner also teaches an ELISA for *E. canis* that uses an *E. canis* antigen derived from mouse J774.A1-infected cells. See page 241, first column, first full paragraph. Waner, therefore, teaches <u>entire cells</u> or <u>whole proteins</u> as assay antigens. As such, Waner cannot teach, suggest, or inherently disclose the specific, individual polypeptides shown in SEQ ID NOs:3-7. Furthermore, Waner does not

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identify the polypeptide fragments to be of any particular diagnostic use. There is no teaching in Waner, directly or inherently, that would direct one of skill in the art to the particular defined sequences of SEQ ID NOs:3-7 or the specified variants for any reason. Warner does not teach or suggest that polypeptides of SEQ ID NOs:3-7 would be useful as individual polypeptides apart from entire *E. canis* infected cells or entire proteins. Warner provides no recognition or suggestion that the distinct polypeptides shown in SEQ ID NOs:1-3 or any other polypeptide fragments would be of diagnostic use.

Importantly, the specification teaches that:

> Indirect immunofluorescense assays (IFA) and enzyme-linked immunosorbent assays (ELISA) are frequently used as aids in the diagnosis of these diseases. These assays measure or otherwise detect the binding of anti-*Ehrlichia* antibodies from a patient's blood, plasma, or serum to infected cells, cell lysates, or purified *Ehrlichia* proteins. However, currently known assays for detecting anti-*Ehrlichia* antibodies or fragments thereof are severely limited in usefulness because of sensitivity and specificity issues directly related to the impure nature of the *Ehrlichia* antigen used in these tests. See page 2, line 21 through page 3, line 2 (emphasis added).

The instant invention provides highly purified reagents for the detection *Ehrlichia*, that is, polypeptides of about 18 to about 20 amino acids, whereas Waner teaches the use of reagents comprising whole infected cells or whole *E. canis* proteins derived from infected cells. The Waner reagents are impure reagents, which the instant specification teaches are of limited usefulness due to the sensitivity and specificity issues. Additionally, the claimed device provides greater sensitivity and specificity than the reagents used in Waner (*i.e.,* DH82 cells that are heavily infected with *E. canis* and *E. canis* antigen derived from mouse J774.A1-infected cells). See Example 1 of

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specification (teaching that assays that use a synthetic peptide of the invention were more sensitive and specific than assays that use native *E. canis* antigens, *i.e.,* partially purified *E. canis* antigens); declaration of Dr. Chandrashekar, paragraphs 2-4 and 6 (of record, copy attached in Appendix C). Therefore, the claimed devices differ from those of Waner because they provide greater sensitivity and specificity than those described in Waner.

The fact that a certain characteristic may occur or be present in a prior art reference is not sufficient to establish the inherency of that characteristic. *In re Rijckaert*, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993); *In re Oelrich*, 212 USPQ 323, 326 (CCPA 1981). "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.' " *In re Robertson*, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) (citations omitted); M.P.E.P. §2112.01. "In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy,* 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) (emphasis in original); M.P.E.P. §2112.01.

The Office has not provided a basis in fact and/or technical reasoning to show that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art. Waner does not teach or suggest the use of polypeptide fragments in the

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detection of *Ehrlichia*. Nor has the Office Action alleged that the mixture of protein antigens or whole cell antigens taught by Waner would be fragmented in any way.

Waner does not anticipate claims 39-42 because Waner does not teach, suggest, or inherently disclose each and every element of claims 39-42. Applicants respectfully request withdrawal of the rejection.

III. Claims 21-24 and 39-42 are not anticipated by Cadman under 35 U.S.C. §102(a).

Claims 21-24 and 39-42 stand rejected under 35 U.S.C. §102(a) as allegedly anticipated by Cadman. Applicants respectfully traverse the rejection.

The Office Action asserts that Cadman teaches an indirect fluorescent assay (IFA) for *E. canis* and that one of skill in the art could reasonable conclude that the *E. canis* polypeptides used by Cadman are substitution variants of one of the polypeptides of SEQ ID NOs3-7. The Office Action also asserts that one of skill in the art would reach this conclusion because the Applicants have not provided a side-by-side comparison to show that the Cadman device differs from the claimed invention. The Office Action furthermore asserts that one of skill in the art could conclude that the "polypeptides" taught by Cadman are substitution variants of the claimed polypeptides because the "polypeptides" of Cadman are used on a device (a cross blot dot apparatus/nitrocellulose paper) to detect antibodies to *E. canis*.

Claims 21-24 recite devices containing one or more polypeptides consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and amino acid substitution variants thereof that specifically bind to an anti-*Ehrlichia* antibody. Claims 39-42 recite devices containing one or more

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polypeptides selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, that specifically bind to an anti-*Ehrlichia* antibody.

Initially it should be noted that SEQ ID NOs:3-7 are derived from *E. chaffeensis* (see Table 1 of specification) and that Cadman does not teach any *E. chaffeensis* sequences. As such Cadman cannot anticipate devices containing SEQ ID NOs:3-7.

Additionally, Cadman does not teach or suggest the use of distinct polypeptides as shown in SEQ ID NOs:1-7 and the claimed specified variants. That is, Cadman does not teach or suggest an about 18 to about 20 amino acid polypeptide of SEQ ID NO:1-7 or the specified amino acid substitution variants recited in claims 21-24. Cadman teaches an IFA for *E. canis* that uses DH82 cells which are heavily infected with *E. canis* as an antigen. See Cadman, first column, fourth paragraph. Cadman also teaches a dot-blot enzyme linked immunoassay (DBELIA) for *E. canis* that uses an *E. canis* antigen purified from infected DH82 cells. See Cadman, first column, fifth paragraph. As such, Cadman teaches the use of whole *E. canis* infected cells or whole proteins purified from *E. canis* infected cells in the disclosed assays. Therefore, Cadman does not teach, suggest, or inherently disclose the specific, individual polypeptides shown in SEQ ID NOs:1-7 and does not identify the polypeptide fragments to be of any particular diagnostic use. There is no teaching in Cadman, directly or inherently, that would direct one of skill in the art to the particular, defined sequences of SEQ ID NOs:1-7 or specified amino acid substitution variants of claims 21-24 for any reason. Cadman does not teach or suggest that polypeptides of SEQ ID NOs:1-7 or specified variants would be useful as individual polypeptides apart from entire *E. canis* infected cells or entire proteins.

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Cadman provides no recognition or suggestion that the distinct polypeptides shown in SEQ ID NOs:1-7, specified amino acid variants, or any other polypeptide fragments would be of diagnostic use.

Additionally, Cadman teaches the use of impure reagents, *i.e.,* whole *E. canis* infected cells or whole proteins derived from infected cells. The instant invention, however, provides highly purified reagents that are much more sensitive and specific. See e.g., specification page 2, line 21 through page 3, line 2. For instance, Example 1 demonstrates that assays that use a synthetic peptide were more sensitive and specific than assays that use native *E. canis* antigens, *i.e.*, partially purified *E. canis* antigens. Furthermore, the claimed device provides greater sensitivity and specificity than the reagents used in Cadman (*i.e.*, DH82 cells which are heavily infected with *E. canis* and *E. canis* antigens purified from infected DH82 cells). See declaration of Dr. Chandrashekar, paragraphs 2-3 and 5-6 (of record, attached as Appendix C). Therefore, the claimed devices differ from those of Cadman because they provide greater sensitivity and specificity than those described in Cadman.

Finally, as discussed above for Waner, the Office has not provided a basis in fact and/or technical reasoning to show that the allegedly inherent characteristic (*i.e.*, SEQ ID NOs:1-7) necessarily flows from the teachings of the applied prior art. Cadman does not teach or suggest the use of polypeptide fragments in the detection of *Ehrlichia.* Nor has that Office Action alleged that the mixture of protein antigens or whole cell antigens in Cadman would be fragmented in any way.

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Cadman does not teach each and every element of the claimed invention and therefore does not anticipate the claimed invention. Applicants respectfully request withdrawal of the rejection.

IV. Claims 21-24 are not anticipated by Rikihisa under 35 U.S.C. §102(b).

Claims 21-24 stand rejected under 35 U.S.C. §102(b) as allegedly anticipated by Rikihisa. Applicants respectfully traverse the rejection.

Claims 21-24 recite devices containing one or more polypeptides consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and amino acid substitution variants thereof that specifically bind to an anti-*Ehrlichia* antibody.

The Office Action incorrectly asserts that the claims are drawn to a composition and article of manufacture consisting essentially of an isolated polypeptide shown in SEQ ID NOs:1-7 and amino acid substitution variants thereof that specifically bind to an anti-Ehrlichia antibody. The Office Action asserts that Rikihisa teaches an amino acid variant of SEQ ID NO:7 that has 85% identity to SEQ ID NO:7 and asserts that one of skill in the art could reasonably conclude that the Rikihisa *E. canis* polypeptides are variants of SEQ ID NO:7 since Rikihisa teaches that the invention embraces non-naturally occurring allelic forms or derivatives of outer membrane proteins.

Rikihisa does not teach or suggest an element of the claims, that is, one or more polypeptides consisting of SEQ ID NOs:1-7. Therefore, the Rikihisa reference cannot anticipate the claims. The claims recite an *E. canis* polypeptide fragment. The polypeptide fragments are useful, *inter alia*, to detect the presence of anti-*Ehrlichia*

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antibodies. The polypeptide fragments can be used as reagents in assays that provide greater sensitivity than the reagents taught in Rikihisa (*i.e.*, whole, recombinant proteins). See declaration of Dr. Chandrashekar, paragraphs 2-3 and 6-7 (of record, attached as Appendix C). The specification also teaches that:

> currently known assays for detecting anti-*Ehrlichia* antibodies or fragments thereof are severely limited in usefulness because of sensitivity and specificity issues directly related to the impure nature of the *Ehrlichia* antigen used in these tests. See page 2, line 25 through page 3, line 1.

The Office appears to assert that the full-length proteins taught by Rikihisa would read on claimed polypeptide fragments. However, the addition of amino acids to the polypeptides so they encompass the whole proteins of Rikihisa would materially affect the characteristics of the polypeptides. That is, use of full-length proteins would result in assays that are less sensitive than those disclosed in the instant specification. As such, the claims cannot be read so that the whole proteins of Rikihisa read on the claimed fragments.

The Office Action additionally asserts that Rikihisa teaches the polypeptides of the invention at Figure 19B. However, Rikihisa teaches a whole *E. canis* P30 protein ("[t]he p30 polynucleotide encodes a P30 protein of *E. canis* having a molecular weight of about 28.8 kDa and an amino acid sequence which is at least 85% homologous to the amino acid sequence shown in FIG. 19B." See page 7, first full paragraph). Figure 19B shows the amino acid sequence of a whole *E. canis* P30 protein.

The instant claims recite devices containing one or more polypeptides consisting of SEQ ID NOs:1-7 and amino acid substitution variants thereof that specifically bind to an anti-*Ehrlichia* antibody. That is, the instant claims recite particular fragments of a *E.*

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canis and *E. chaffeensis* proteins. Rikihisa does not teach or suggest the claimed polypeptide fragments removed from their position within a larger protein, such as P30. The claimed polypeptides are not disclosed in Rikihisa as separate and distinct polypeptides, but merely as a part of a larger, entire protein sequence. There is no teaching in Rikihisa that would direct one of skill in the art to select the particular claimed polypeptides for any reason. As such, Rikihisa does not teach or suggest the claimed devices.

The Office appears to rely on an inherency theory to support the rejection. However, the Office has not provided a basis in fact and/or technical reasoning to show that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art. Rikihisa does not teach or suggest the use of polypeptide fragments in devices and in particular does not teach or suggest the particular fragments shown in SEQ ID NOs:1-7. Nor has that Office Action alleged that the whole recombinant protein antigens in Rikihisa would be fragmented in any way.

Rikihisa does not anticipate claims 21-24 because Rikihisa does not teach, suggest, or inherently disclose each and every element of claims 21-24. Applicants respectfully request withdrawal of the rejection.

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Summary

On the basis of the foregoing and in view of the arguments presented herein, reversal of each and every rejection is appropriate.

Respectfully submitted,

Date: 7/23/04 By: _____

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APPENDIX A

Pending Claims

1. (Withdrawn) A composition of matter comprising an isolated polypeptide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and variants thereof.

2. (Withdrawn) The composition of claim 1, further comprising a carrier.

3. (Withdrawn) A method of detecting presence of antibodies to Ehrlichia comprising:

(a) contacting one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof, with a test sample suspected of comprising antibodies to Ehrlichia, under conditions that allow polypeptide/antibody complexes to form;

(b) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that antibodies to Ehrlichia are present in the test sample.

4. (Withdrawn) The method of claim 3, further comprising contacting the complexes of step (a) with an indicator reagent comprising a signal generating compound that generates a measurable signal prior to the performance of step (b).

5. (Withdrawn) The method of claim 3, wherein the presence of antibodies to *Ehrlichia canis* are detected.

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6. (Withdrawn) The method of claim 3, wherein the presence of antibodies to *Ehrlichia chaffeensis* are detected.

7. (Withdrawn) The method of claim 3, wherein the antibodies are fragments of antibodies.

8. (Withdrawn) The method of claim 3 wherein the amount of antibody in a test sample is determined.

9. (Withdrawn) The method of claim 3, wherein the polypeptide is attached to a substrate.

10. (Withdrawn) The method of claim 3, wherein the polypeptide provided is shown in SEQ ID NO:1.

11. (Withdrawn) The method of claim 3, wherein the polypeptide provided is shown in SEQ ID NO:2.

12. (Withdrawn) The method of claim 3, wherein the polypeptide provided is shown in SEQ ID NO:3.

13. (Withdrawn) The method of claim 3, wherein the polypeptide provided is shown in SEQ ID NO:4.

14. (Withdrawn) The method of claim 3, wherein the polypeptide provided is shown in SEQ ID NO:5.

15. (Withdrawn) The method of claim 3, wherein the polypeptide provided is shown in SEQ ID NO:6.

16. (Withdrawn) The method of claim 3, wherein the polypeptide provided is shown in SEQ ID NO:7.

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17. (Withdrawn) The method of claim 3, wherein the one or more polypeptides are provided in a multimeric form.

18. (Withdrawn) The method of claim 3, wherein the test sample is a biological sample obtained from a mammal.

19. (Withdrawn) The method of claim 18, wherein the mammal is selected from the group consisting of humans and dogs.

20. (Withdrawn) The method of claim 3 wherein the method comprises an assay selected from the group of assays consisting of a reversible flow chromatographic binding assay, an enzyme linked immunosorbent assay, a western blot assay, and an indirect immunofluorescense assay.

21. (Previously Presented) A device containing one or more polypeptides consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and amino acid substitution variants thereof that specifically bind to an anti-*Ehrlichia* antibody.

22. (Previously Presented) The device of claim 21, further comprising instructions for use of the one or more polypeptides for the identification of an *Ehrlichia* infection in a mammal.

23. (Previously Presented) The device of claim 22, wherein the instructions for use indicate that the identification of an *Ehrlichia* infection is done using a method of detecting presence of antibodies to *Ehrlichia* comprising:

(a) contacting one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ

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ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and amino acid substitution variants thereof that specifically bind to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form;

(b) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present.

24. (Previously Presented) The device of claim 22, wherein the *Ehrlichia* infection is caused by *Ehrlichia canis* or *Ehrlichia chaffeensis*.

25. (Withdrawn) An article of manufacture comprising packaging material and, contained within the packaging material, one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof.

26. (Withdrawn) The article of manufacture of claim 25 wherein the packaging material comprises a label that indicates that the one or more polypeptides can be used for the identification of Ehrlichia infection in a mammal.

27. (Withdrawn) The article of manufacture of claim 26, wherein the identification of an Ehrlichia infection is done using a method of detecting presence of antibodies to Ehrlichia comprising:

(a) contacting one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof, with a test sample

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suspected of comprising antibodies to Ehrlichia, under conditions that allow polypeptide/antibody complexes to form;

(b) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that an Ehrlichia infection is present.

28. (Withdrawn) The article of manufacture of claim 26, wherein the Ehrlichia infection is caused by *Ehrlichia canis* or *Ehrlichia chaffeensis.*

29. (Withdrawn) A method of diagnosing an Ehrlichia infection in a mammal comprising:

(a) obtaining a biological sample from a mammal suspected of having an Ehrlichia infection;

(b) contacting one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof, with the biological sample under conditions that allow polypeptide/antibody complexes to form;

(c) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that the mammal has an Ehrlichia infection.

30. (Withdrawn) The method of claim 29 further comprising contacting the complexes of step (b) with an indicator reagent comprising a signal generating compound that generates a measurable signal prior to the performance of step (c).

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31. (Withdrawn) The method of claim 29, wherein the Ehrlichia infection is caused *by Ehrlichia canis.*

32. (Withdrawn) The method of claim 29, wherein the Ehrlichia infection is caused by *Ehrlichia chaffeensis.*

33. (Withdrawn) The method of claim 29, wherein the mammal is a human or a dog.

34. (Withdrawn) A monoclonal antibody that specifically binds to at least one epitope of an *Ehrlichia canis* or *Ehrlichia chaffeensis* polypeptide, said polypeptide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

35. (Canceled)

36. (Canceled)

37. (Canceled)

38. (Canceled)

39. (Previously Presented) A device containing one or more polypeptides selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, that specifically bind to an anti-*Ehrlichia* antibody.

40. (Previously Presented) The device of claim 39, further comprising instructions for use of the one or more polypeptides for the identification of an *Ehrlichia* infection in a mammal.

41. (Previously Presented) The device of claim 39, wherein the instructions for use indicate that the identification of an *Ehrlichia* infection is done using a method of detecting presence of antibodies to *Ehrlichia* comprising:

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(a) contacting one or more polypeptides selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, that specifically bind to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form;

(b) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present.

42. (Previously Presented) The device of claim 39, wherein the *Ehrlichia* infection is caused by *Ehrlichia canis* or *Ehrlichia chaffeensis*.

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APPENDIX B

Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SEQ ID NO:1	K	S	T	V	G	V	F	G	L	K	H	D	W	D	G	S	P	I	L	K
SEQ ID NO:2	N	T	T	T	G	V	F	G	L	K	Q	D	W	D	G	A	T	I	K	D
SEQ ID NO:3	N	T	T	V	G	V	F	G	L	K	Q	N	W	D	G	S	A	I	S	N
SEQ ID NO:4	N	P	T	V	A	L	Y	G	L	K	Q	D	W	N	G	V	S	A		
SEQ ID NO:5	N	T	T	V	G	V	F	G	I	E	Q	D	W	D	R	C	V	I	S	
SEQ ID NO:6	N	P	T	V	A	L	Y	G	L	K	Q	D	W	E	G	I	S	S		
SEQ ID NO:7	N	T	T	T	G	V	F	G	L	K	Q	D	W	D	G	S	T	I	S	

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PATENT

UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 00-1278)

In the Application of:

Lawton, et al.

Serial No.: **09/765,739**

Filed: **January 18, 2001**

Art Unit: 1645

Examiner: V. Ford

For: **Compositions and Methods for Detection of *Ehrlichia canis* and *Ehrlichia chaffeensis* Antibodies**

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Ramaswamy Chandrashekar, am a research scientist for IDEXX Laboratories, Inc., the entire Assignee of U.S. Pat. Appl. Ser. Nos. 09/765,739, 10/054,354, and 10/054,647. I have earned a Ph.D. in Biochemistry and M.S. in Zoology. I have performed research and development in the field of sero-diagnostics for veterinary bacterial pathogens, including, for example, *Ehrlichia* ssp. and *Streptococcus equi* for over five years. In addition, I have performed research and development in the field of sero-diagnostics for nematode infections in both humans and animals for over 20 years. I am an author of over 70 scientific publications in the field of diagnosis and prevention of nematode infections. A copy of my Curriculum Vitae is attached.

2. Waner *et al., (J. Vet. Diagn. Invest.*, Vol. 12, pp. 240-244, 2000), Cadman *et al., (The Veterinary Record,* 135, 362), Rikihisa (WO 99/13720), and other references

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EXHIBIT
tabbies
C

that teach or suggest the use of entire *E. canis* or *E. chaffeensis* infected cells, whole (*i.e.*, non-fragmented) *E. canis* or *E. chaffeensis* proteins, including mixtures of whole proteins, natural whole proteins, or whole recombinant proteins, to, *e.g.*, detect *Ehrlichia,* do not teach or suggest polypeptides of SEQ ID NOs:1-7 to, *e.g.*, detect *Ehrlichia.* As explained in the specifications of the above-mentioned patent applications, entire *E. canis or E. chaffeensis* infected cells, or whole (i.e., non-fragmented) *E. canis* or *E. chaffeensis* proteins, including mixtures of whole proteins, natural whole proteins or whole recombinant proteins are impure reagents, which are of limited usefulness in sero-diagnosis due to sensitivity and specificity issues. For instance, Example 1 of the 09/765,739 application demonstrates that assays that use SEQ ID NOs:1 and 2 were more sensitive and specific than assays that use partially purified *E. canis* antigens. *See e.g.*, paragraph spanning page 20 and 21 of the 09/765,739 application. The partially purified *E. canis* antigens were obtained from *E. canis* organisms grown in tissue culture and partially purified by differential centrifugation and column chromatography. These partially purified *E. canis* antigens were therefore, mixtures of whole proteins.

3. Assays for detecting anti-*Ehrlichia* antibodies or fragments as described by Waner, Cadman, Rikihisa, and others are severely limited in usefulness because of sensitivity and specificity issues directly related to the impure nature of the *Ehrlichia* antigen used in these tests. *See e.g.*, page 2, line 21 through page 3, line 2 of the 09/765,739 specification (emphasis added). The instant inventions provide highly purified reagents for the detection *Ehrlichia*, that is, polypeptides of about 18-20 amino acids. The use of SEQ ID NOs:1-7 instead of the impure reagents described above, to for

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example, detect *Ehrlichia* provide distinct advantages such as greater sensitivity and specificity in sero-diagnostic assays.

4. Waner teaches that the disclosed ELISA assay for detection of *E. canis* closely correlates to the "gold standard" IFA test. *See e.g.*, p. 243, left col. first full paragraph; page 243, right col., first and second paragraph; page 240, right col., first full paragraph.

5. Cadman teaches that the disclosed dot-blot enzyme linked immunoassay (DBELIA) had a sensitivity of 92% and a specificity of 96% when compared to the IFA. See page 135, paragraph spanning columns. Cadman states that the "study showed the DBELIA to be as sensitive and specific as IFA for the detection of antibodies to *E. canis*." See last paragraph.

6. The polypeptides claimed in the instant application have a sensitivity of 98.5% and a specificity of 100% when compared to western blot analysis. Western blot analysis is more sensitive and more specific than IFA analysis. The IFA had, at one time, been considered the "gold standard" for sero-diagnosis of *Ehrlichia*. However, western analysis is more sensitive and more specific than IFA analysis, which uses whole cells as the antigen resulting in cross-reactivity, specificity, and sensitivity issues. The IFA disclosed in the instant invention had a sensitivity of 88% and a specificity of 0%. The polypeptides of the instant invention perform better than the "gold standard" IFA in this study. The Waner and Cadman assays, however, perform only as well as the IFA. Therefore, one of skill in the art could reasonably conclude that the polypeptides of the instant invention perform better, *i.e.*, provide more sensitive and specific results in sero-diagnostic assays, than the Waner and Cadman assays.

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7. Rikihisa teaches the use of recombinant, whole proteins to detect *Ehrlichia* antibodies. Rikihisa does not disclose the sensitivity or specificity of the whole, recombinant proteins in sero-diagnostic assays. However, Ohashi *et al.* (J. Clin. Microbiol. 36:2671 (1998)) (copy attached) teaches that dot blot assays performed with whole *E. canis* rP30 antigen to detect *E. canis* were as sensitive as an IFA assay, specificity was not examined in this study. See page 2678, right column, first full paragraph. The instant invention provides peptides (SEQ ID NOs:1-7) that can provide results that are more sensitive than IFA assays. Therefore, one of skill in the art could reasonably conclude that the peptides of the instant invention are more sensitive and more specific than the antigens reported in Waner, Cadman, and are more sensitive than the antigens reported in Rikihisa.

8. The pure reagents described in the instant inventions have additional advantages as compared to the impure reagents described in Waner, Cadman, and Rikihisa. For example, in experiments performed at IDEXX Laboratories mixtures of SEQ ID NOs:1 and 2 showed no cross-reactivity to *Borrelia burgdorferi, A. phagocytophilum*, and uninfected canine serum. See Table 1.

Table 1.

Peptide	**# of Samples**	**Canine Serum**	**Reactivity**
Mixtures of SEQ ID NO:1 and SEQ ID NO:2	157	Uninfected	0/157
	81	*E. canis*	81/81
	166	*Borrelia burgdorferi*	0/166
	29	*A. phagocytophilum*	0/29

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9. I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 02/13/2004 By: _____

Ramaswamy Chandrashekar

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PROFESSIONAL EXPERIENCE

IDEXX LABORATORIES, WESTBROOK, MAINE

RESEARCH SCIENTIST-II, RAPID ASSAY GROUP, INFECTIOUS DISEASES R&D (2002-PRESENT)

Leading a group responsible for R&D of Point Of Care of Testing devices for companion animals.

HESKA CORPORATION, FORT COLLINS, COLORADO

SENIOR SCIENTIST AND GROUP LEADER, Diagnostic Research (1998-2002)

Management of molecular- and immuno-diagnostic group. Supervised a group of five Scientists (including two Ph.D. Scientists). Designed, developed, and evaluated molecular and immunodiagnostic assays for the diagnosis of parasitic and infectious agents in companion animals. Developed, optimized, and validated immunoassays for cancer markers. Project management: primary person responsible for the management and coordination of projects under aggressive time-lines. Responsible for transfer of assays, documentation and SOPs. Interacted extensively with groups with in the company (process and regulatory groups) and outside the organization (external scientific/technical collaborations with both academia and industry).

ACCOMPLISHMENTS

Participated in the research and/or development of the following *Heska* products and reagents:

- ***Heska™* Canine *Ehrlichia* sp. PCR Test** (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- ***Heska™* Feline ImmuCheck™ Assay** (Vaccine Titer Assay) (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- ***Heska™ Equine Streptococcus equi* PCR Test** (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- ***Heska™* Canine Heartworm Antigen Test** (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- ***Heska™* SoloStep™ CH** (USDA Licensed Canine Heartworm Diagnostic POCT).
- ***Heska™* SoloStep™ FH** (USDA Licensed Feline Heartworm Diagnostic POCT).
- Seven patents pending.
- Identified several potential diagnostic targets for a fecal diagnostic kit for intestinal helminth infections.

SENIOR SCIENTIST, Nematode Vaccines and Diagnostics (1995-1998)

Management of vaccine antigen discovery and testing group. Supervised one post-doctoral and five associate scientists. Conducted basic research on the biology/ biochemistry/immunology/proteomics of parasitic nematodes to identify potential vaccine antigens. Molecular cloning and expression of recombinant nematode vaccine antigens. Designed and coordinated several animal studies for vaccine efficacy trials (dogs and cats). Antigen discovery research for heartworm diagnostics.

ACCOMPLISHMENTS

- Identified and characterized of over ten potential vaccine candidate nematode antigens against heartworm infections for clinical trials in dogs/cats.
- Five potential recombinant antigens identified by proteomics and cloned for diagnostic evaluation in antibody detection ELISA for feline heartworm infections.
- 13 Patents issued and one pending.

WASHINGTON UNIVERSITY, ST. LOUIS, MISSOURI

RESEARCH ASSISTANT PROFESSOR, Dept. of Medicine and Molecular Microbiology (1995)
RESEARCH INSTRUCTOR, Dept. of Medicine and Molecular Microbiology (1991-1995)

Responsible for immunodiagnostic assay development and vaccine discovery studies in parasitic nematode infections. Field evaluation of diagnostic tests in Egypt. Supervised science technicians.

ACCOMPLISHMENTS

- Developed recombinant antigen-based antibody ELISAs for the diagnosis of human nematode infections. Evaluation of both assays extensively in field studies in Africa.
- Developed a monoclonal antibody-based antigen-based diagnostic assay for human onchocerciasis (river blindness) to detect circulating parasite antigens both in blood and urine.
- Developed a recombinant antigen-based immunoblot assay for diagnosis of Histoplasmosis.
- Identified and tested several candidate recombinant vaccine antigens in animal model of filarial nematode infections.
- One Patent issued and one pending.

NATIONAL/INTERNATIONAL SCIENTIFIC ACTIVITIES

- *Invited Participant-WHO:* Filariasis Scientific Working Group (UNDP/World Bank/World Health Organization), 1991, 1994. "Protective immunity studies in Onchocerciasis.
- *Chairperson:* Chaired the scientific session in Filarial Biology. Joint Annual Meeting of the American Society of Tropical Medicine and Hygiene and The American Society of Parasitologists, Atlanta, Georgia. October 31-November 4, 1993.
- *Advisor-WHO:* Special Program for Research & Training in Tropical Diseases (TDR), World Health Organization, 1993, 1994. Transferred technology from lab research to product development.
- *Consultant and collaborating scientist,* Epidemiology and Control of Vector Borne Diseases in the Middle East (Egypt-Israel-USA) (USAID/NIH), 1990-1994. Transferred immunodiagnostic assays for lymphatic filariasis from laboratory to field for evaluation studies in Egypt.
- *Collaborating Scientist,* Participated in protective immunity studies in human filariasis-*International Collaborations in Infectious Disease Research Project (NIH)* to study immunity to filariasis in humans with a longitudinal study of carefully defined populations in a highly endemic region of Egypt, 1994-1995.

POST-DOCTORAL RESEARCH ASSOCIATE (Jewish Hospital of St. Louis at Washington University) (1988-1991)

Identified, characterized and generated monoclonal antibodies to circulating parasitic nematode antigens; Developed antigen detection and recombinant antigen-based antibody assays for human infectious diseases. Participated in a Recombinant DNA Technology Workshop conducted by the *New England Biolabs* and Smith College, Northampton (1991). Supervised a science technician.

CIBA-GEIGY RESEARCH CENTER, BOMBAY, INDIA

SENIOR RESEARCH ASSISTANT (1986-1988)

RESEARCH ASSISTANT (1980-1986)

EDUCATION

Ph.D., Biochemistry - CIBA-GEIGY Research Center, and University of Bombay, India.

MS., Zoology - University of Madras, Madras, India.

HONORS AND AWARDS

National Science Talent Search Scholarship, NCERT, New Delhi, India.
Joshua Gold Medal for best under graduate student.
Joshua Gold Medal and Aaron award for best post graduate student.
National Merit Scholarship, Government of India.

PROFESSIONAL MEMBERSHIPS

American Society of Tropical Medicine and Hygiene.
American Association for the Advancement of Science.
American Society of Parasitologists.
American Society for Microbiologists.

PUBLICATIONS

(SEE ADDENDUM)

Peer-reviewed-64; Invited-7

ADDENDUM

PUBLICATIONS

PEER-REVIEWED

1. Rao RR, Marathe MR, **Chandrashekar R**, Subrahmanyam D: Ocular filarial infections in *Mastomys natalensis* with *Litomosoides carinii* and *Brugia pahangi*. *Indian J Parasitol* 1983;7:57-60.
2. Reddy AB, Rao UR, **Chandrashekar R**, Shrivastava R, Subrahmanyam D: Comparative efficacy of some benzimidazoles and amoscanate (Go. 9333) against experimental filarial infections. *Tropenmed Parasitol* (Germany) 1983; 34:259-262.
3. **Chandrashekar R**, Rao UR, Rajasekariah GR, Subrahmanyam D: Separation of viable microfilariae free of blood cells on Percoll gradients. *J Helminthol* 1984;58:69-70.
4. **Chandrashekar R**, Rao UR, Subrahmanyam D, Hopper K, Nelson DS, King M: *Brugia pahangi*: Serum-dependent cell-mediated reactions to sheathed and exsheathed microfilariae. *Immunology* 1984;53:411-417.
5. **Chandrashekar R**, Rao UR, Rajasekariah GR, Subrahmanyam D: Isolation of microfilariae from blood on iso-osmotic Percoll gradients. *Indian J Med Res* 1984;79:497-501.
6. **Chandrashekar R**, Rao UR, Subrahmanyam D: Effect of diethylcarbamazine on serum dependent cell-mediated reactions to microfilariae in vitro. *Tropenmed Parasitol* (Germany) 1984;35:177-182.
7. **Chandrashekar R**, Parab PB, Subrahmanyam D: The effect of p-amino-benzoic acid and folic acid on the development of infective larvae of *Brugia malayi* in *Aedes aegypti*. *Acta Trop* (Switzerland) 1984;41:61-67.
8. Reddy AB, **Chandrashekar R**, Rao UR, Subrahmanyam D: Microfilarial periodicity in *Mastomys natalensis*. *J Helminthol* 1984;58:117-121.
9. Hopper K, Subrahmanyam D, Gregory J, Nelson DS, Rao UR, **Chandrashekar R**: Mechanisms of ADCC of rat cells to microfilariae: effects of metabolic inhibitors and electron microscopic observations. *Asian Pac J Allergy Immunol* 1984;2:17-21.
10. **Chandrashekar R**, Rao UR, Subrahmanyam D, Hopper K, Nelson DS, King M: Immune reactions to exsheathed microfilariae of *Litomosoides carinii*. *Indian J Med Res* 1985;81:260-268.
11. **Chandrashekar R**, Rao UR, Subrahmanyam D: Sharing of antigens among filarial species in antibody-dependent cell-mediated cytotoxicity. *J Biosci* (India) 1985;9:191-196.
12. **Chandrashekar R**, Rao UR, Parab PB, Subrahmanyam D: *Brugia malayi*: Cell-mediated immune reactions to microfilariae. *Southeast Asian J Trop Med Public Health* 1985;16:15-21.
13. **Chandrashekar R**, Rao UR, Subrahmanyam D: Serum-dependent cell-mediated immune reactions to *Brugia pahangi* infective larvae. *Parasite Immunol* 1985;7:633-641.
14. **Chandrashekar R**, Rao UR, Arab PB, Subrahmanyam D: *Brugia malayi*: Rat-cellular interactions with infective larvae mediated by complement. *Exp Parasitol* 1986;62:362-369.
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Cloning and Characterization of Multigenes Encoding the Immunodominant 30-Kilodalton Major Outer Membrane Proteins of *Ehrlichia canis* and Application of the Recombinant Protein for Serodiagnosis

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A 30-kDa major outer membrane protein of *Ehrlichia canis*, the agent of canine ehrlichiosis, is the major antigen recognized by both naturally and experimentally infected dog sera. The protein cross-reacts with a serum against a recombinant 28-kDa protein (rP28), one of the outer membrane proteins of a gene (*omp-1*) family of *Ehrlichia chaffeensis*. Two DNA fragments of *E. canis* were amplified by PCR with two primer pairs based on the sequences of *E. chaffeensis omp-1* genes, cloned, and sequenced. Each fragment contained a partial 30-kDa protein gene of *E. canis*. Genomic Southern blot analysis with the partial gene probes revealed the presence of multiple copies of these genes in the *E. canis* genome. Three copies of the entire gene (*p30*, *p30-1*, and *p30a*) were cloned and sequenced from the *E. canis* genomic DNA. The open reading frames of the two copies (*p30* and *p30-1*) were tandemly arranged with an intergenic space. The three copies were similar but not identical and contained a semivariable region and three hypervariable regions in the protein molecules. The following genes homologous to three *E. canis* 30-kDa protein genes and the *E. chaffeensis omp-1* family were identified in the closely related rickettsiae: *wsp* from *Wolbachia* sp., *p44* from the agent of human granulocytic ehrlichiosis, *msp-2* and *msp-4* from *Anaplasma marginale*, and *map-1* from *Cowdria ruminantium*. Phylogenetic analysis among the three *E. canis* 30-kDa proteins and the major surface proteins of the rickettsiae revealed that these proteins are divided into four clusters and the two *E. canis* 30-kDa proteins are closely related but that the third 30-kDa protein is not. The *p30* gene was expressed as a fusion protein, and the antibody to the recombinant protein (rP30) was raised in a mouse. The antibody reacted with rP30 and a 30-kDa protein of purified *E. canis*. Twenty-nine indirect fluorescent antibody (IFA)-positive dog plasma specimens strongly recognized the rP30 of *E. canis*. To evaluate whether the rP30 is a suitable antigen for serodiagnosis of canine ehrlichiosis, the immunoreactions between rP30 and the whole purified *E. canis* antigen were compared in the dot immunoblot assay. Dot reactions of both antigens with IFA-positive dog plasma specimens were clearly distinguishable by the naked eye from those with IFA-negative plasma specimens. By densitometry with a total of 42 IFA-positive and -negative plasma specimens, both antigens produced results similar in sensitivity and specificity. These findings suggest that the rP30 antigen provides a simple, consistent, and rapid serodiagnosis for canine ehrlichiosis. Cloning of multigenes encoding the 30-kDa major outer membrane proteins of *E. canis* will greatly facilitate understanding pathogenesis and immunologic study of canine ehrlichosis and provide a useful tool for phylogenetic analysis.

Canine ehrlichiosis is caused by *Ehrlichia canis*, an obligatory intracellular bacterium. It was described originally in Algeria in 1935 (7), and it has now been reported throughout the world and at higher frequency in tropical and subtropical regions (13, 15, 32). Canine ehrlichiosis is characterized by fever, depression, anorexia, and weight loss in the acute phase, with laboratory findings of thrombocytopenia and hypergammaglobulinemia (3, 9). A subclinical phase follows the acute phase (5, 12, 28). In the chronic phase, in addition to the clinical signs and laboratory findings of the acute phase, hemorrhages, epistaxis, edema, and hypotensive shock may occur, which are often exacerbated by superinfection with other organisms (3, 9, 16).

Among several protein antigens of *E. canis*, the proteins in the 30-kDa range were shown to be dominant antigens and consistently recognized by sera from both experimentally and naturally infected dogs in Western blot analysis (14, 25, 26). The proteins of *E. canis* immunologically cross-react with *Ehrlichia chaffeensis* major antigens in the 30-kDa range (25). These *E. canis* and *E. chaffeensis* proteins were found to be major outer membrane proteins (OMPs) (22). Analysis of a 28-kDa major OMP (P28) gene of *E. chaffeensis*, one of the 30-kDa-range antigens, and its gene copies revealed that these proteins are encoded by a polymorphic multigene family (22). The rabbit serum against a recombinant *E. chaffeensis* P28 protein cross-reacted with the 30-kDa protein of *E. canis* (22).

Dot immunoblot assaying has been developed for serodiagnosis of several infectious agents (4, 10, 11, 30). The advantages of the assay are that an expensive instrument is not required and the interpretation of the results is easy, since positive and negative reactions can be distinguished by the naked eye. However, to be used as the antigen, purification of the organism from infected cells is essential, since *E. canis* is an obligate intracellular bacterium. Purification of *E. canis* is time-consuming and expensive, and serial passages of *E. canis*

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in the cell culture may produce batch-to-batch variations. Although, no genes of *E. canis* other than the 16S rRNA gene have thus far been identified, preparation of a recombinant major antigen is expected to greatly improve the serodiagnosis of *E. canis* infection.

In this study, three genes encoding the 30-kDa OMPs from the *E. canis* genome were identified. All were found to be homologous and phylogenetically characterized. A recombinant protein of *E. canis* which was expressed as a fusion protein was found to be highly antigenic. The dot immunoblot assay was developed with the recombinant *E. canis* protein.

MATERIALS AND METHODS

Organisms and purification. *E. canis* Oklahoma and *E. chaffeensis* Arkansas were cultivated in the DH82 dog macrophage cell line and purified by Percoll density gradient centrifugation (22) or Sephacryl S-1000 column chromatography (26).

PCR, cloning, and expression. The sequences of two forward primers, FECH1 and FECH2, were 5'-CGGGATCCGAATTCGG(A/T/G/C)AT(A/T/C)AA(T/C)GG(A/T/G/C)AA(T/C)TT(T/C)TA-3' and 5'-CGGGATCCGAATTCTA(T/C)AT(A/T)AG(T/C)GG(A/T/G/C)AA(A/G)TA(T/C)ATG-3', corresponding to amino acid positions 6 to 12 and positions 12 to 18, respectively, of the mature 28-kDa protein (P28) of *E. chaffeensis* (22). These primers have a 14-bp sequence (underlined) at the 5' end to create an *Eco*RI site and a *Bam*HI site for insertion into an expression vector. The sequence of a reverse primer, REC1, was 5'-ACCTAACTTTCCTTGGTAAG-3', complementary to the DNA sequence corresponding to amino acid positions 185 to 191 of the mature P28 of *E. chaffeensis* (22).

Genomic DNA of *E. canis* was isolated from Percoll gradient-purified organisms as described elsewhere (22). PCR amplification was performed by using a Perkin-Elmer Cetus DNA Thermal Cycler (model 480). The 0.6-kb products were amplified with both primer pairs, FECH1-REC1 and FECH2-REC1, and were cloned in the pCRII vector of a TA cloning kit (Invitrogen Co., San Diego, Calif.). The clones obtained by FECH1-REC1 and FECH2-REC1 were designated pCRII*p30* and pCRII*p30a*, respectively. Both strands of the insert DNA were sequenced by a dideoxy termination method with an Applied Biosystems 373 DNA sequencer.

For expression, the 0.6-kb fragment was excised from the clone pCRII*p30* by *Eco*RI digestion, ligated into *Eco*RI site of a pET29a expression vector, and amplified in *Escherichia coli* BL21(DE3)pLys (Novagen, Inc., Madison, Wis.). The clone (designated pET29*p30*) produced a fusion protein with 35-amino-acid and 21-amino-acid sequences carried from the vector at the N and C termini, respectively.

For purification of a recombinant P30 fusion protein (rP30), the cultivated clone was harvested at 4 h after induction with β-D-thiogalactopyranoside. The recombinant protein in the clone pET29*p30* was enriched in the pellet by three cycles of centrifugation of the lysate after disruption of the transformant by freezing-thawing and sonication. The final pellet was used as a partially purified rP30 antigen. Affinity-purified rP30 protein was obtained by chromatography with His-Bind Resin (Novagen, Inc.). Briefly, after preparation of the partially purified rP30 antigen, the insoluble protein was extracted with binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), including 6 M urea. After being applied to a Ni^+-conjugated column, the recombinant protein was eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 6 M urea. The refolding of the purified protein was achieved by sequential dialysis in 20 mM Tris-HCl (pH 7.9) containing 4 and 2 M urea and finally in 20 mM Tris-HCl buffer only and stored at −80°C until use.

Southern blot analysis. Genomic DNA extracted from the Percoll-purified *E. canis* (200 ng each) was digested with restriction enzymes, electrophoresed, and transferred to a Hybond-N^+ nylon membrane (Amersham, Arlington Heights, Ill.) by a standard method (27). The 0.6-kb DNA inserts containing partial *p30* and *p30a* genes, cloned in pCRII*p30* and pCRII*p30a*, respectively, were separately labeled with [α-^{32}P]dATP by the random primer method with a kit (Amersham), and each labeled fragment was used for Southern blot analysis as a DNA probe. Hybridization was performed at 60°C in Rapid Hybridization buffer (Amersham) for 20 h. The nylon sheet was washed in 0.1× SSC (1× SSC containing 0.15 M sodium chloride and 0.015 M sodium citrate) with 1% sodium dodecyl sulfate (SDS) at 55°C, and the hybridized probes were exposed to Hyperfilm (Amersham) at −80°C.

Cloning and sequencing of 30-kDa protein gene copies from the *E. canis* genomic DNA. The *Hin*dIII DNA fragment, which was detected by genomic Southern blot analysis as described above, was inserted into pBluescript II KS(+) vectors, and the recombinant plasmids were introduced into *E. coli* DH5α. By using the colony hybridization method (27), two positive clones which contained ehrlichial DNA fragments of 3.6 and 7.3 kb were isolated with the ^{32}P-labeled inserts of pCRII*p30* and pCRII*p30a* as probes, respectively. DNA sequencing was performed with suitable synthetic primers by the dideoxy termination method described above.

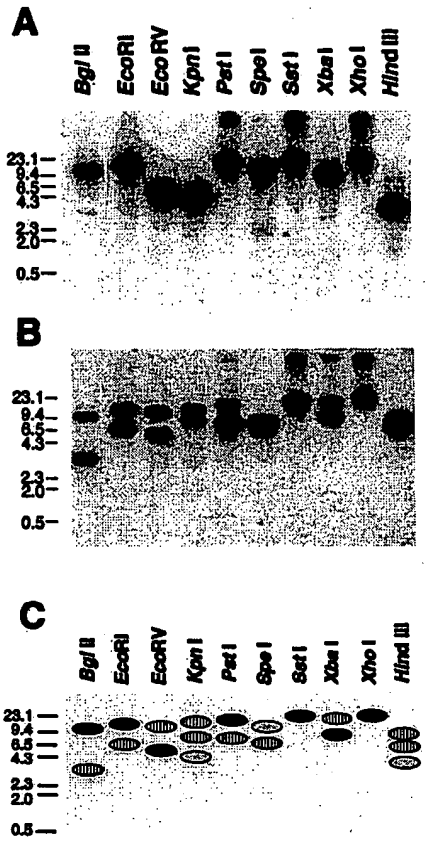


FIG. 1. Genomic Southern blot analysis of *E. canis* DNA with the partial *p30* gene probe (A) and with the partial *p30a* gene probe (B) and schematic representation of the blotting patterns (C). Numbers indicate molecular sizes in kilobases. Filled dots, bands hybridized with both p30 and p30a probes; striped dots, bands hybridized with p30a probe alone; lightly shaded dots, bands hybridized with p30 probe alone.

Sequence analysis. DNA and amino acid sequences were analyzed with the programs DNASIS (Hitachi Software Engineering America, Ltd., San Bruno, Calif.) and DNASTAR (DNASTAR Inc., Madison, Wis.). The amino acid sequences were aligned by using the CLUSTAL method in the DNASTAR program. Phylogenetic analysis was performed by using the PHYLIP software package (version 3.5) (8). An evolutionary distance matrix, generated by using the Kimura formula in the program PROTDIST in the package, was used for construction of a phylogenetic tree by using the unweighted pair-group method of analysis (8). The data were examined by using parsimony analysis (PROTPARS in the PHYLIP). A bootstrap analysis was carried out to investigate the stability of randomly generated trees by using SEQBOOT and CONSENSE in the same package.

Dog plasma and mouse serum. Totals of 34 and 8 dog blood samples with heparin or EDTA were obtained from the Southwest Veterinary Diagnostic Center (Phoenix, Ariz.) and at the Ohio State University Veterinary Teaching Hospital, respectively. All blood specimens collected were centrifuged at 250 × *g* for 5 min, and the plasma samples were used for this study. For Western blot analysis, these plasma samples were preabsorbed three times with pET29a-transformed *E. coli* at 4°C overnight prior to use. For preparation of the mouse anti-rP30 serum, a male mouse (BALB/c) was intraperitoneally immunized a total of four times at 10-day intervals, once with an equal mixture of the affinity-purified rP30 (30 μg of protein) and Freund's complete adjuvant (Sigma) and three times with an equal mixture of the protein (30 μg) and Freund's incomplete adjuvant. The mouse was sacrificed 7 days after final immunization, and the serum was prepared from blood collected from the heart.

IFA and Western blot analysis. Indirect fluorescent antibody assays, (IFA) and Western blot analysis were performed by a procedure described elsewhere (25). Fluorescein isothiocyanate-conjugated goat anti-dog immunoglobulin G (IgG; Organon Teknika Co., Durham, N.C.) and peroxidase-conjugated affinity-purified anti-dog IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) were used at dilutions of 1:200 for IFA and 1:2,000 for Western blot analysis, respectively, as secondary antibodies.

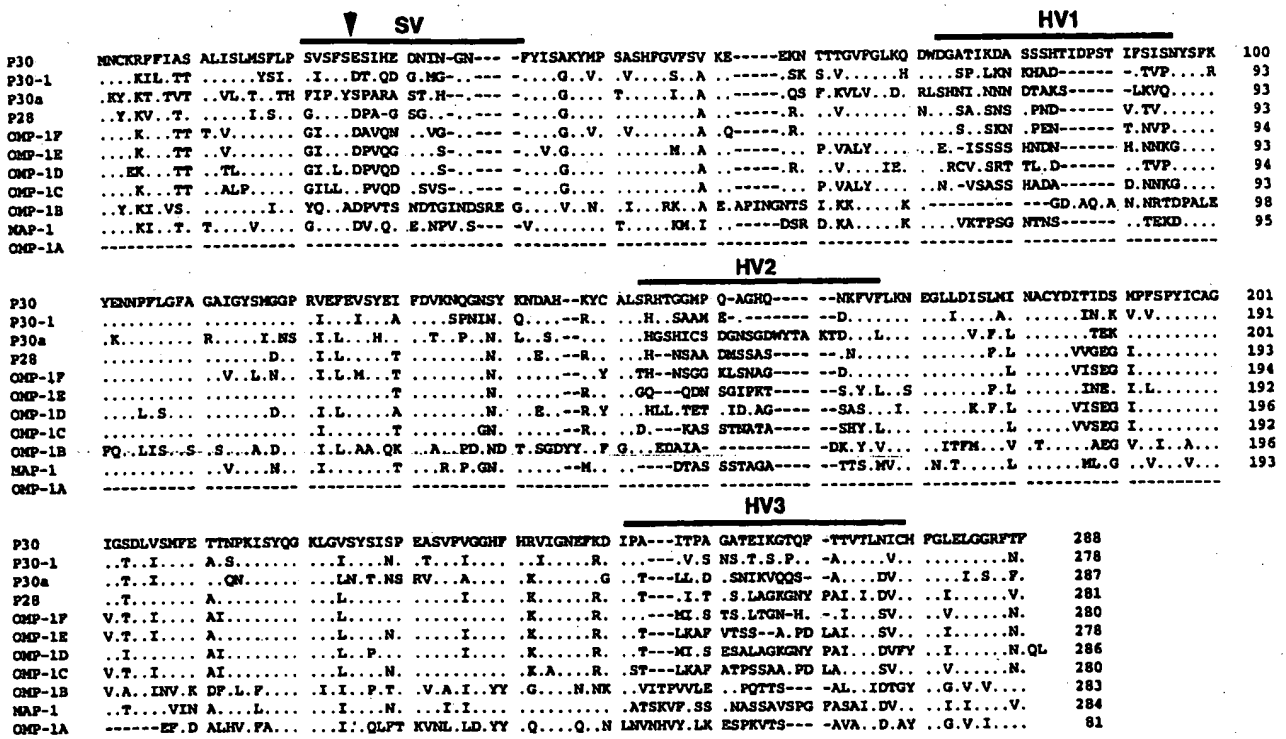
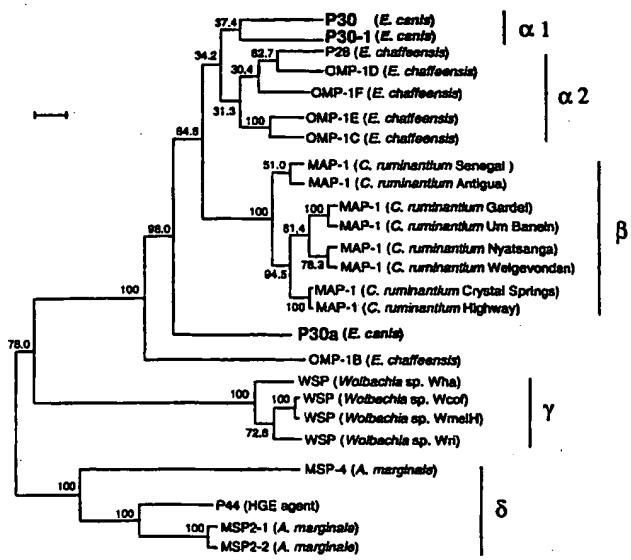


FIG. 2. Amino acid sequence alignment of P30, P30-1, and P30a of *E. canis*, seven members of *E. chaffeensis omp-1* multigene family (P28 and OMP-1A to OMP-1F), and MAP-1 of *C. ruminantium* (Senegal strain). The sequences of the *E. chaffeensis omp-1* gene family and MAP-1 are from the reports of Ohashi et al. (22) and Van Vliet et al. (31), respectively. Aligned positions of identical amino acids with P30 of *E. canis* are indicated by dots. Gaps (indicated by dashes) were introduced for optimal alignment of all proteins. Bars indicate an SV and three HVs (HV1, -2, and -3). The arrowhead indicate the putative cleavage site of the signal peptide.

Dot immunoblot assay. Protein concentrations of purified *E. canis* and recombinant rP30 antigens were determined by a bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as a standard. These antigens in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.4], 150 mM NaCl) were adsorbed onto a nitrocellulose membrane by using a dot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.), blocked for 30 min with TBS containing 2% milk, air dried, and stored at −20°C until use. For immunoassays, the antigen bound to a nitrocellulose strip was incubated with the plasma samples, which were diluted 1:1,000 in TBS containing 2% milk for 1 h at room temperature. After being washed three times with TBS containing 0.05% Tween 20 (T-TBS), the strip was incubated with peroxidase-conjugated affinity-purified anti-dog IgG (Kirkegaard) at a dilution of 1:2,000 in TBS containing 2% milk. After being washed with T-TBS, the antibody-bound dot was detected by immersing the strip in a developing solution (0.3% 3,3′-diaminobenzidine tetrahydrochrolide [Nacalai Tesque, Inc., Kyoto, Japan] and 0.05% hydrogen peroxide in 70 mM sodium acetate [pH 6.2]). The color intensity was analyzed by using background correction in image analysis software (ImageQuaNT program; Molecular Dynamics, Sunnyvale, Calif.).

GenBank accession number. The DNA sequences of the *p30*, *p30a*, and *p30-1* genes of *E. canis* have been assigned GenBank accession numbers AF078553, AF078555, and AF078554, respectively.



RESULTS

Cloning and sequencing of three 30-kDa protein gene copies of *E. canis*. Two 0.6-kb DNA fragments containing partial *p30*

FIG. 3. Phylogenetic classification among P30, P30-1, and P30a of *E. canis* and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Evolutionary distance values were determined by the method described by Kimura, and the tree was constructed by the unweighted pair-group method of analysis. Scale bar indicates 10% divergence in amino acid sequences. Bootstrap values from 100 analyses are shown at the branch points of the tree. Bars with symbols indicate representative clusters. The GenBank accession numbers of the major OMP gene sequences of the organisms used in the analysis are as follows: P28 (*E. chaffeensis*), U72291; OMP-1B to OMP-1F (*E. chaffeensis*), AF021338; MAP-1 (*C. ruminantium* Senegal strain), I40882, MAP-1 (*C. ruminantium* Antigua strain), U50830; MAP-1 (*C. ruminantium* Gardel strain), U50832; MAP-1 (*C. ruminantium* Um Banein strain), U50835; MAP-1 (*C. ruminantium* Nyatsanga strain), U50834; MAP-1 (*C. ruminantium* Welgevonden strain), U49843; MAP-1 (*C. ruminantium* Crystal Springs strain), U50831; MAP-1 (*C. ruminantium* Highway strain), U50833; WSP (*Wolbachia* sp. Wha strain), AF020068; WSP (*Wolbachia* sp. Wcof strain), AF020067; WSP (*Wolbachia* sp. WmelH strain), AF020066; WSP (*Wolbachia* sp. Wri strain), AF020070; MSP-4 (*A. marginale*), Q07408; MSP2-1 (*A. marginale*), U07862; MSP2-2 (*A. marginale*), U36193; and P44 (HGE agent), AF059181.

TABLE 1. Similarities among amino acid sequences of *E. canis* P30, P30-1, and P30a; *E. chaffeensis omp-1* family (OMP-1B to OMP-1F and P28); *C. ruminantium* MAP-1; *Wolbachia* spp. WSP; HGE agent P44; and *A. marginale* MSP-4, MSP2-1, and MSP2-2

Protein	% Amino acid sequence similarity and evolutionary distance for the following proteins[a]:										
	P30	P30-1	P30a	P28	OMP-1F	OMP-1E	OMP-1D	OMP-1C	OMP-1B	MAP-1 (Senegal)	MAP-1 (Antigua)
P30		80.2	70.8	80.6	80.5	78.6	77.8	77.5	63.2	75.4	76.2
P30-1	0.38628		71.6	79.8	81.7	78.7	78.3	77.3	63.2	74.7	75.6
P30a	0.60811	0.60559		73.9	72.1	73.3	71.2	72.1	58.8	67.2	67.8
P28	0.36288	0.40582	0.50899		85.7	82.3	86.3	81.1	63.6	76.4	77.5
OMP-1F	0.37862	0.36209	0.59907	0.27551		83.4	84.9	83.0	63.2	75.4	75.8
OMP-1E	0.41426	0.42866	0.52142	0.35465	0.32640		81.7	90.1	63.4	76.8	78.1
OMP-1D	0.45193	0.46724	0.61591	0.25793	0.28867	0.36288		81.5	63.2	73.5	74.5
OMP-1C	0.45426	0.48329	0.57469	0.39823	0.34577	0.18285	0.37688		62.4	76.0	77.5
OMP-1B	0.89214	0.87276	0.99793	0.81397	0.83501	0.82982	0.84498	0.89516		62.7	63.2
MAP-1 (Senegal)	0.50490	0.51605	0.76041	0.46987	0.50383	0.46987	0.57453	0.50564	0.92668		93.9
MAP-1 (Antigua)	0.47614	0.50899	0.74635	0.46755	0.52220	0.46096	0.57153	0.48952	0.88842	0.09122	
MAP-1 (Gardel)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.13499	0.11546
MAP-1 (Crystal Springs)	0.55702	0.53478	0.78883	0.52220	0.56563	0.49693	0.59089	0.53368	0.93601	0.13657	0.14142
MAP-1 (Highway)	0.52891	0.52047	0.76041	0.49443	0.54364	0.46987	0.57594	0.50564	0.93601	0.12383	0.12856
MAP-1 (Nyatsanga)	0.50593	0.49693	0.76544	0.49196	0.53368	0.46755	0.57296	0.48952	0.91855	0.13077	0.11963
MAP-1 (Um Banein)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.12658	0.11963
MAP-1 (Welgevonden)	0.52629	0.50383	0.74708	0.49877	0.53368	0.47419	0.60290	0.48952	0.92979	0.16080	0.14519
WSP (Wha)	1.57097	1.66864	1.78274	1.59949	1.50435	1.38174	1.61950	1.45510	1.41776	1.58338	1.48404
WSP (Wcof)	1.46262	1.62571	1.62571	1.55195	1.40877	1.29961	1.60271	1.41762	1.33110	1.55897	1.53089
WSP (WmelH)	1.48165	1.64952	1.64952	1.54244	1.39991	1.31514	1.59304	1.43572	1.34750	1.54961	1.49206
WSP (Wri)	1.46435	1.66864	1.70518	1.55687	1.46526	1.27219	1.57654	1.39076	1.32111	1.53292	1.47465
P44	1.77884	1.84928	2.04164	1.56146	1.74020	1.64702	1.64376	1.64702	1.64566	1.57894	1.63909
MSP-4	1.37226	1.39399	1.62744	1.38660	1.45473	1.36494	1.45413	1.47002	1.34294	1.23482	1.31702
MSP2-1	1.50323	1.53992	1.90757	1.40230	1.59474	1.53455	1.40877	1.50435	1.52758	1.53992	1.54847
MSP2-2	1.52476	1.53992	1.87540	1.40230	1.57132	1.53455	1.40877	1.50435	1.55019	1.51796	1.52616

[a] Values in the upper right half are percent amino acid sequence similarities; those in the lower left half are evolutionary distances.

and *p30a* genes, amplified by PCR, were cloned and sequenced as described in Materials and Methods. The 0.6-kb DNA, cloned in pCRII*p30*, had an open reading frame (ORF) of 579 bp encoding a 193-amino-acid protein with a molecular mass of 21,175 Da. Another 0.6-kb fragment, cloned in pCRII*p30a*, had an ORF of 564 bp encoding a 188-amino-acid protein with a molecular mass of 21,042 Da. The DNA and predicted amino acid sequences of the partial *p30a* gene were similar but not identical to those of the partial *p30* gene. Genomic Southern blot analysis of *E. canis* digested with several restriction enzymes revealed one and two DNA fragments which could strongly hybridize to the partial *p30* and *p30a* gene probes, respectively (Fig. 1). These restriction enzymes used do not cut within the *p30* and *p30a* gene probes, and, therefore, the result with the *p30a* probe indicates that another gene homologous to the *p30a* is present in the *E. canis* genome. In *Bgl*II, *Eco*RI, and *Pst*I digestion, the *p30* probe hybridized with the upper band of the two *p30a*-hybridized bands. In *Eco*RV and *Xba*I digestion, the *p30* probe hybridized with the lower band of the two *p30a*-hybridized bands. In *Kpn*I, *Spe*I, and *Hin*dIII digestion, the *p30* probe hybridized with one or two bands that were different from the *p30a*-hybridized bands.

Two DNA fragments of 3.6 and 7.3 kb were cloned by colony hybridization with the probes described above from the *Hin*dIII-digested genomic DNA of *E. canis*. Sequencing revealed a complete ORF of 864 bp for the *p30* gene in the 3.6-kb fragment and a complete ORF of 861 bp for *p30a* gene in the 7.3-kb DNA fragment. An additional ORF of 921 bp was found in the 3.6-kb DNA. The DNA sequence of the ORF (designated *p30-1*) was also similar but not identical to those of the *p30* and *p30a* genes. There are two potential start codons in the *p30-1* gene sequence. By comparison with the N-terminal amino acid sequences of *p30* and *p30a* genes, we chose a second ATG as a start codon for phylogenetic analysis. The coding region is 834 bp. The *p30-1* and *p30* genes were tandemly arranged with an intergenic space of 355 bp in the 3.6-kb fragment like the *E. chaffeensis omp-1* family (22). In addition to the result of the genomic Southern blot analysis, this finding showed that at least four homologous genes (*p30*, *p30-1*, *p30a*, and a gene homologous to *p30a*) exist in the *E. canis* genome, suggesting that these genes of *E. canis* are also encoded by a polymorphic multigene family as is the case with *E. chaffeensis* (22).

Structure of proteins encoded by *E. canis* multigenes. Three complete gene copies (*p30*, *p30-1*, and *p30a*) encode 278- to 288-amino-acid proteins with molecular masses of 30,485 to 31,529 Da. The 25-amino-acid sequence at the N termini of P30, P30-1, and P30a (encoded by *p30*, *p30-1*, and *p30a*, respectively) is predicted to be a signal peptide, as described previously (22). The molecular masses of the mature proteins calculated based on the predicted amino acid sequences are 28,750 Da for *p30*, 27,727 Da for *p30-1*, and 29,132 Da for *p30a*.

The predicted amino acid sequences of *E. canis* P30, P30-1, and P30a showed high similarity with those of members in the *E. chaffeensis omp-1* gene family (22) and that of major antigen protein 1 (MAP-1) of *Cowdria ruminantium* (31). These organisms are also serologically cross-reactive (6, 17, 18, 19, 20). The alignment of amino acid sequences of these proteins revealed substitutions or deletions of one or several contiguous amino acid residues throughout the molecules (Fig. 2). The significant differences in sequences among the proteins are observed in the regions designated SV (semivariable region) and HV (hypervariable region). Computer analysis for hydropathy revealed that protein molecules predicted for three *E. canis* gene copies contain alternative hydrophilic and hydrophobic motifs which are characteristic of typical transmembrane proteins. HV1 and HV2 were located in the hydrophilic regions (data not shown).

TABLE 1—*Continued*

% Amino acid sequence similarity and evolutionary distance for the following proteins:													
MAP-1 (Gardel)	MAP-1 (Crystal Springs)	MAP-1 (Highway)	MAP-1 (Nyatsanga)	MAP-1 (Um Banein)	MAP-1 (Welgevonden)	WSP (Wha)	WSP (Wcof)	WSP (WmelH)	WSP (Wri)	P44	MSP-4	MSP2-1	MSP2-2
76.4	74.5	75.4	75.8	76.4	75.2	44.4	44.6	44.4	44.4	19.5	45.6	27.8	27.4
74.7	73.9	74.3	74.7	74.7	74.5	44.0	45.1	44.8	44.6	20.5	47.6	29.3	29.1
67.6	65.9	66.5	66.7	67.6	67.2	41.5	43.2	42.9	42.5	19.5	43.1	24.2	24.2
75.8	74.5	75.4	75.2	75.8	74.9	44.0	44.8	44.8	44.6	22.5	46.9	29.7	29.5
74.5	73.3	73.9	73.9	74.5	73.9	44.6	45.9	45.9	45.3	21.1	46.2	27.8	27.8
76.2	75.4	76.2	76.0	76.2	75.8	45.7	46.9	46.7	46.9	22.0	47.5	28.2	28.0
74.1	73.1	73.5	73.3	74.1	72.4	43.6	44.2	44.2	44.2	22.0	46.0	29.9	29.7
75.8	74.5	75.4	75.6	75.8	75.6	45.3	46.1	45.9	46.1	22.0	46.6	28.6	28.4
63.6	63.2	63.2	63.2	63.6	62.9	45.5	45.1	44.8	45.5	19.1	45.8	26.9	26.5
91.4	90.7	91.4	91.6	91.8	90.1	44.6	45.1	45.1	45.1	21.8	48.8	28.0	28.0
91.8	90.7	91.4	91.6	91.6	90.3	44.8	45.1	45.3	45.3	21.8	48.0	28.0	28.0
	92.2	92.8	94.9	99.6	93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.12928		98.9	93.1	92.4	93.1	43.4	43.4	43.4	43.2	20.0	46.1	26.7	26.7
0.11692	0.01764		93.7	93.1	93.7	43.8	43.8	43.8	43.6	20.2	46.5	27.2	27.2
0.08788	0.11285	0.10076		94.5	95.4	43.8	43.8	43.8	43.8	20.5	46.7	28.0	27.8
0.00693	0.12514	0.11285	0.09570		93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.11966	0.11285	0.10076	0.08014	0.11966		44.2	44.0	44.0	44.0	20.2	46.5	27.8	27.6
1.51972	1.73099	1.65953	1.64538	1.51972	1.58048		86.1	86.1	90.3	12.5	42.5	22.9	22.7
1.47157	1.59304	1.53089	1.55897	1.47157	1.52893	0.27243		98.3	90.9	13.6	42.1	24.0	24.0
1.46262	1.58338	1.52153	1.54961	1.46262	1.51972	0.26757	0.03029		90.7	13.6	42.3	23.8	23.8
1.44526	1.64362	1.57654	1.53292	1.44526	1.50279	0.18429	0.17605	0.17691		13.6	43.2	24.0	23.8
1.62813	1.74020	1.71093	1.68253	1.62813	1.71093	2.06354	2.15803	2.14440	2.09032		25.7	45.5	45.2
1.33120	1.35101	1.30992	1.31112	1.33120	1.33120	1.72157	1.96007	1.90199	1.72157	1.20170		35.6	34.9
1.50996	1.57836	1.53304	1.46817	1.50996	1.48884	1.70865	1.79325	1.81891	1.72741	0.83164	1.20880		95.6
1.50996	1.55543	1.51116	1.46817	1.50996	1.48884	1.70865	1.75923	1.78382	1.72741	0.84284	1.23930	0.05064	

Phylogenetic relationship among the three *E. canis* 30-kDa proteins and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Recently, several major OMP genes which are closely related to the *E. canis* 30-kA protein have been cloned from rickettsiae (2, 21–24, 31, 34). The phylogenetic tree consisting of 25 major OMPs of the organisms including P30, P30-1, and P30a of *E. canis* was constructed from the estimated evolutionary distances (Fig. 3). The overall pattern of the tree reflects the result based on 16S rRNA gene sequence analysis of the rickettsiae. The 23 representatives, except for *E. canis* P30a and *E. chaffeensis* OMP-1B, are divided into four groups as follows: *E. canis* and *E. chaffeensis*, group α; *C. ruminantium*, group β; *Wolbachia* sp., group γ; and the agent of human granulocytic ehrlichiosis (HGE) and *Anaplasma marginale*, group δ. Group α formed a subcluster of *E. canis* P30 and P30-1 (group α1), which was separated from another subcluster composed of five *E. chaffeensis* OMPs (group α2). The similarities between P30 and P30-1 of *E. canis* in group α1, between groups α1 and α2, between groups α1 and β, between groups α1 and γ, and between groups α1 and δ were 80.2%, 77.3 to 80.6%, 73.9 to 76.4%, 44.0 to 45.1%, and 19.5 to 47.6%, respectively (Table 1). On the other hand, *E. canis* P30a and *E. chaffeensis* OMP-1B were far from group α and were located between groups β and γ. The similarities between *E. canis* P30a and group α1, between P30a and group α2, between P30a and group β, between P30a and group γ, and between P30a and group δ were 70.8 to 71.6%, 71.2 to 73.9%, 65.9 to 67.8%, 41.5 to 43.2%, and 19.5 to 43.1%, respectively.

Expression of the *E. canis p30* gene. The clone pET29*p30* produced a 249-amino-acid fusion protein with a molecular mass of 27,316 Da (Fig. 4A). The recombinant protein (rP30) with minimum *E. coli* contamination detectable was obtained in the pellet by centrifugation of the lysate of the transformant (Fig. 4B [partially purified antigen]). The rP30 protein further purified by affinity chromatography from this preparation had a single band on SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 4B [affinity-purified antigen]). The immunoreactions of *E. canis* rP30 with a total of 42 clinical dog plasma specimens were examined. The IgG-IFA titers of 29 plasma samples were 1:20 to 1:10,480. The remaining plasma samples were IFA negative (<1:20). Western blot analysis revealed that all IFA-positive plasma samples recognized the partially purified rP30 fusion protein (27 kDa) and a 30-kDa protein of

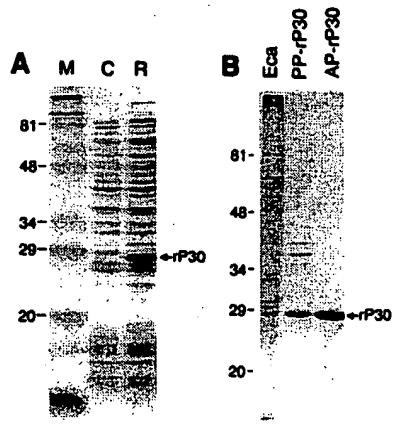


FIG. 4. SDS-PAGE profiles of a recombinant clone expressing P30 of *E. canis* (A) and the purified recombinant protein (B). Gels were stained with Coomassie blue. Lanes: M, molecular size markers; C, pET29-transformed *E. coli* (negative control); R, pET29*p30*-transformed *E. coli* (recombinant); Eca, purified *E. canis*; PP-rP30, partially purified rP30 fusion protein of *E. canis*; and AP-rP30, affinity-purified rP30 fusion protein. The recombinant rP30 protein is indicated by the arrow. The numbers on the left of each panel indicate molecular masses in kilodaltons.

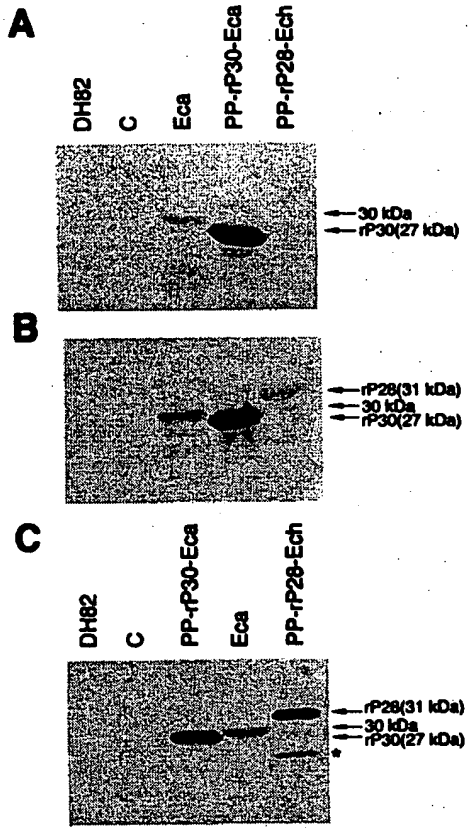


FIG. 5. Western blot analysis with clinical dog plasma with canine ehrlichiosis (A and B) and mouse anti-rP30 serum (C). (A) Dog plasma with a 1:40 IFA titer against *E. canis*; (B) dog plasma with a 1:1,280 IFA titer. Lanes: DH, DH82 dog macrophage cell (negative control); C, a pET29-transformed *E. coli* (negative control); Eca, purified *E. canis* (reactive 30-kDa protein is indicated by arrows in each panel); PP-rP30-Eca, a partially purified rP30 fusion protein (27 kDa) of *E. canis*; and PP-rP28-Ech, a partially purified rP28 fusion protein (31 kDa) of *E. chaffeensis* (22). Another smaller reactive band which may be a degradation product of rP28 of *E. chaffeensis* is indicated by an asterisk.

purified *E. canis* (one of the blots is shown in Fig. 5A), but none of 13 negative plasma samples reacted with any proteins of partially purified rP30 and purified *E. canis* (data not shown). Eight of the 29 positive plasma samples reacted weakly with recombinant P28 fusion protein (rP28 [31 kDa]) of *E. chaffeensis* (22) (one of the blots is shown in Fig. 5B), but the remaining plasma samples did not. A mouse anti-rP30 serum which was prepared by immunization with the affinity-purified antigen reacted with the rP30 antigen, a 30-kDa protein of purified *E. canis*, and an rP28 of *E. chaffeensis* (Fig. 5C). Another smaller band which was observed with *E. chaffeensis* rP28 may be a degradation product of rP28 (asterisk in Fig. 5C), since the plasma sample did not react with *E. coli* proteins. These results showed that rP30 of *E. canis* is highly antigenic and that the antigenic epitope is expressed.

Dot immunoblot assay with the purified whole organism antigen and the recombinant antigen. (i) Optimum amount of antigen per dot. Western blot analysis and dot immunoblot assaying in the preliminary experiments supported the interpretation that there are no significant differences between affinity-purified and the partially purified rP30 in specificity and sensitivity (data not shown). If partially purified recombinant protein is suitable for serodiagnosis, it will be more cost-effective. By dot immunoblot assaying we examined in detail whether partially purified rP30 is suitable as an antigen for serodiagnosis.

Nitrocellulose strips having serially diluted purified *E. canis* or partially purified rP30 antigen of *E. canis* were reacted at a 1:1,000 dilution with dog plasma samples with different IFA titers against *E. canis*, and the color intensities of the reaction of each dot were compared (Fig. 6). Dots of 0.01 to 1 μg of the purified organisms (Fig. 6A) or dots of 0.025 to 1 μg of rP30 (Fig. 6B) that reacted with positive plasma samples (>1:20 in IFA titer) were clearly distinguishable from those that reacted with negative plasma samples (<1:20) by the naked eye. There was no nonspecific reaction with the negative plasma samples when purified *E. canis* was used as an antigen; however, a weak nonspecific reaction with IFA-negative plasma was observed in dots of 0.25 to 1 μg of partially purified rP30 antigen. Based on these results, the optimum amounts of antigens per dot were determined to be 1 and 0.5 μg for antigen proteins of purified *E. canis* and partially purified rP30, respectively. These results show that the partially purified recombinant protein is apparently sufficient as an antigen for serodiagnosis.

(ii) Optimum dilution of antiserum. The immunoreactivities of plasma at dilutions of 1:300, 1:1,000, and 1:3,000 were examined with nitrocellulose strips of the purified *E. canis* an-

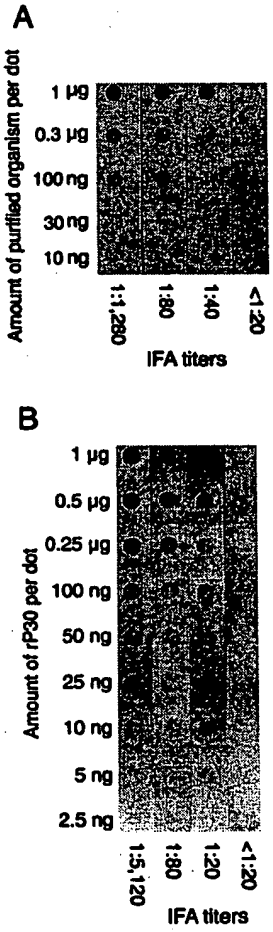


FIG. 6. Optimum amount of antigens for dot blot assaying with purified *E. canis* antigen (A) or partially purified rP30 antigen (B). Purified organism antigen (10 ng to 1 μg) or rP30 antigen (2.5 ng to 1 μg) was blotted onto the nitrocellulose sheet, reacted with each plasma at a 1:1,000 dilution as primary antibody, and reacted with secondary antibody (peroxidase-conjugated affinity-purified anti-dog IgG antibody) at a 1:2,000 dilution.

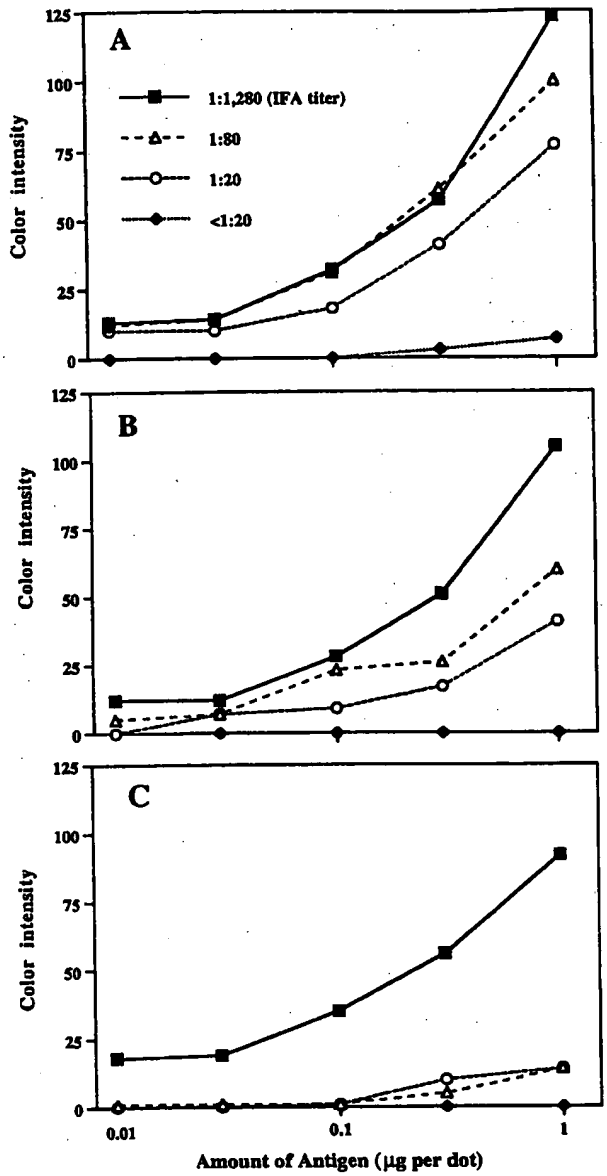


FIG. 7. Optimum plasma dilutions for dot blot assay. Purified *E. canis* antigen was blotted as described in the legend to Fig. 6. The antigens were incubated with plasma at dilutions of 1:300 (A), 1:1,000 (B), and 1:3,000 (C). The plasma samples used were the same as those used for Fig. 6A. The color intensity of each dot was determined by using the image software program (ImageQuaNT).

tigen as shown in Fig. 6A. The color intensity values were plotted in graphs (Fig. 7). At a 1:300 dilution (Fig. 7A), color development occurred in the dots having an antigen greater than 0.3 μg per dot with IFA-negative plasma. At a 1:3,000 dilution (Fig. 7C), color intensities of all plasma samples were low, especially in the case of positive plasma samples with low IFA titers (1:20 and 1:80). At a 1:1,000 dilution (Fig. 7B), positive plasma with even the lowest IFA titer (1:20) was distinguishable from IFA-negative plasma by the naked eye, especially with 1 μg of purified *E. canis* antigen per dot (Fig. 6A). The optimum dilution of plasma for testing was, therefore, 1:1,000.

(iii) Examination of clinical dog plasma with purified *E. canis* and partially purified rP30 antigens. A total of 42 clinical dog plasma samples were examined with 1 μg of purified *E. canis* antigen per dot and 0.5 μg of partially purified rP30 antigen per dot (Fig. 8). The plasma samples with higher IFA titers showed a darker reaction with both native and recombinant antigens. The color intensities between plasma with IFA titers of >1:20 and IFA-negative plasma were clearly distinguishable by the naked eye. The correlation between IFA titers and color intensity values by the dot immunoblot assay was examined (Fig. 9). The maximum color intensity values of 13 IFA-negative plasma samples (<1:20) were zero (background) in the purified *E. canis* antigen and 10 in the rP30 antigen. All 29 IFA-positive plasma samples (>1:20) showed color intensity values of greater than 19 in the purified *E. canis* and 18 in the rP30 antigen. The highest color intensity values were 105 in the purified organism and 114 in the rP30 antigen. In both native and recombinant antigens, color intensity values correlated with IFA titers. The correlation coefficients between IFA titers and color intensities of native and recombinant antigens were 0.71 ($P < 0.001$) and 0.68 ($P < 0.001$), respectively. Therefore, it may be possible to estimate an approximate titer of the test serum or plasma by comparing the color densities with those of serially diluted standard serum or plasma.

DISCUSSION

The availability of recombinant immunodominant major surface proteins of *E. canis* will greatly assist in diagnosis and in understanding of the pathogenesis of this intracellular bacterium, such as invasion of host cells, elicitation of the immune response, and mechanisms of the clinical disease. The 30-kDa protein of *E. canis* was shown to be the immunodominant major OMP, which can be recognized by naturally and experimentally infected dog sera (14, 25, 26). Therefore, the 30-kDa protein is the primary recombinant antigen candidate for use in the serodiagnosis of *E. canis* infection. The present study is the first report of molecular characterization of 30-kDa major OMPs of *E. canis*.

Polymorphic multigene families encoding the major OMPs have been identified in *E. chaffeensis*, the HGE agent, and *A. marginale*, which are closely related to *E. canis* based on 16S rRNA gene sequences. Six copies of the *E. chaffeensis p28* gene (*omp-1* gene family) are tandemly arranged with intergenic spaces (22), while copies of the HGE agent *p44* gene and the *A. marginale msp-2* and *msp-3* genes are distributed widely throughout the genomes (1, 23, 34). In this study, the 30-kDa proteins of *E. canis* were also shown to be encoded by a polymorphic multigene family. The two *E. canis* genes are tandemly arranged with an intergenic space as are members of the *E. chaffeensis omp-1* gene family. Although we demonstrated the presence of four gene copies of 30-kDa *E. canis* proteins in the genome, additional gene copies which are tandemly arranged may exist in three genomic *Hin*dIII DNA fragments which hybridized to *p30* and *p30a* probes. Sequence analysis revealed that the 30-kDa proteins (P30, P30-1, and P30a) of *E. canis* had characteristics of the *E. chaffeensis* OMP-1 family (22) and *C. ruminantium* MAP-1 (31). The *C. ruminantium* MAP-1 has been reported to be cross-reactive to a 27-kDa protein of *E. canis* (19), although it is unknown whether the 27-kDa protein is identical to P30, P30-1, or P30a of *E. canis* in this study. Phylogenetic analysis based on the homologs from the closely related rickettsiae revealed that P30 and P30-1 of *E. canis* are present in the same cluster but that P30a is far from the cluster, suggesting that the multigenes encoding the 30-kDa *E. canis* proteins are widely divergent. Interestingly, in the phylogenetic tree, the 30-kDa *E. canis* proteins, the *E. chaffeensis* OMP-1 family, the HGE agent P44, and *A. mar-*

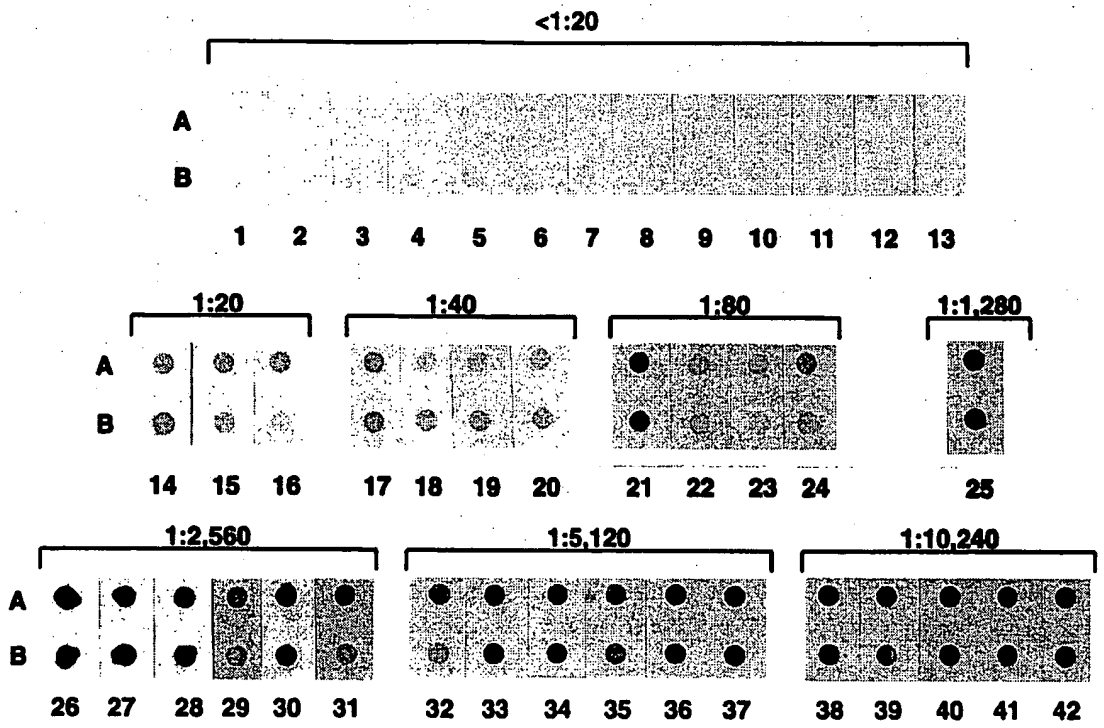


FIG. 8. Reaction profiles of purified *E. canis* antigen (1 μg) (A) and partially purified rP30 antigens (0.5 μg) (B) with 42 plasma samples. Plasma identifications are indicated below each dot. Numbers above brackets indicate the IFA titers of the plasma samples.

ginale MSP-2 are encoded by a polymorphic multigene family as described above. However, *C. ruminantium* MAP-1, *Wolbachia* sp. WSP, and *A. marginale* MSP-4 are encoded by a single gene (2, 21–24, 31). The diversities reported among the *C. ruminantium* MAP-1s and among the *Wolbachia* sp. WSPs are strain variation (2, 24, 31).

Molecular analysis of *E. canis* 30-kDa antigens such as ours is important in understanding the antibody responses of animals, because the antigenic diversity may influence the specificity and sensitivity of the serologic assay. Previously, we observed in the Western blot analysis that acute-phase serum (before 30 days postinoculation) from an *E. canis*-infected dog reacted strongly with a 30-kDa protein but weakly with a 31-kDa protein. However, the reactivity of the chronic-phase serum (after 60 days postinoculation) from the same dog was reversed (strong reaction with the 31-kDa protein and weak reaction with the 30-kDa protein) (14). This might be due to differential expression of the multigene encoding the 30-kDa protein of *E. canis* during infection. Although it is unknown whether the genes of P30, P30-1, and P30a were expressed by *E. canis* in tissue culture or in the infected dog, the recombinant P30 protein constructed in this study expressed the antigenic epitope which can react with all IFA-positive dog plasma samples used, suggesting that the antigenic epitope conserved among the 30-kDa protein gene family is expressed. This strongly supports the idea that rP30 is useful as an antigen for serodiagnosis of canine ehrlichiosis.

For serodiagnosis of canine ehrlichiosis, IFA is widely used. However, a fluorescence microscope and trained personnel are required for this test. Furthermore, cell culture of *E. canis* may produce batch-to-batch variation. A consistent and simple assay that can detect specific antibodies without expensive equipment would be an invaluable aid in serodiagnosis. In the dot immunoblot assay, antibody-positive serum can be distinguished from antibody-negative serum by the naked eye, and if proper color standards are provided, anyone can easily make the final evaluation. The greatest obstacle for the development of this assay is the production of diagnostic antigens sufficient in purity and amount. If recombinant antigens are available, the antigen preparation would be simpler, more consistent, and economical than purified organism antigen preparation. Previously, a dot blot enzyme-linked immunoassay for detecting antibodies to *E. canis* has been reported (4). However, the crude antigens, freed from host cells by freezing-thawing, were used in that study. Neither recombinant antigens nor the purified antigens (such as organisms purified by Sephacryl S-1000 column chromatography) were used. Additionally, that report contains only one page of description without any data. Therefore, we think our dot immunoblot assay using the recombinant 30-kDa antigen of *E. canis* would greatly enhance serodiagnosis of canine ehrlichiosis.

Recognition of the lowest positive IFA titer (1:20) plasma by a dot immunoblot assay with 1 μg or less of protein of the whole organism or the recombinant antigen per dot shows that this assay is as sensitive as IFA. Although the specificity of the test, except for cross-reactivity with *E. chaffeensis*, was not analyzed in this study, as with any other serologic test, dot immunoblot assaying probably cannot distinguish among antigenically cross-reactive members of the tribe *Ehrlichieae*. However, the use of recombinant *E. canis* antigen gave greater sensitivity than the use of recombinant *E. chaffeensis* antigen for serodiagnosis of canine ehrlichiosis. Western blot analysis revealed that 8 of 22 IFA-positive plasma samples slightly cross-reacted with recombinant 28-kDa protein of *E. chaffeensis*. This weak cross-reactivity is not a potential problem for clinics, since treatment is the same for all of the ehrlichial agents.

In dot immunoblot assays of 29 IFA-positive plasma sam-

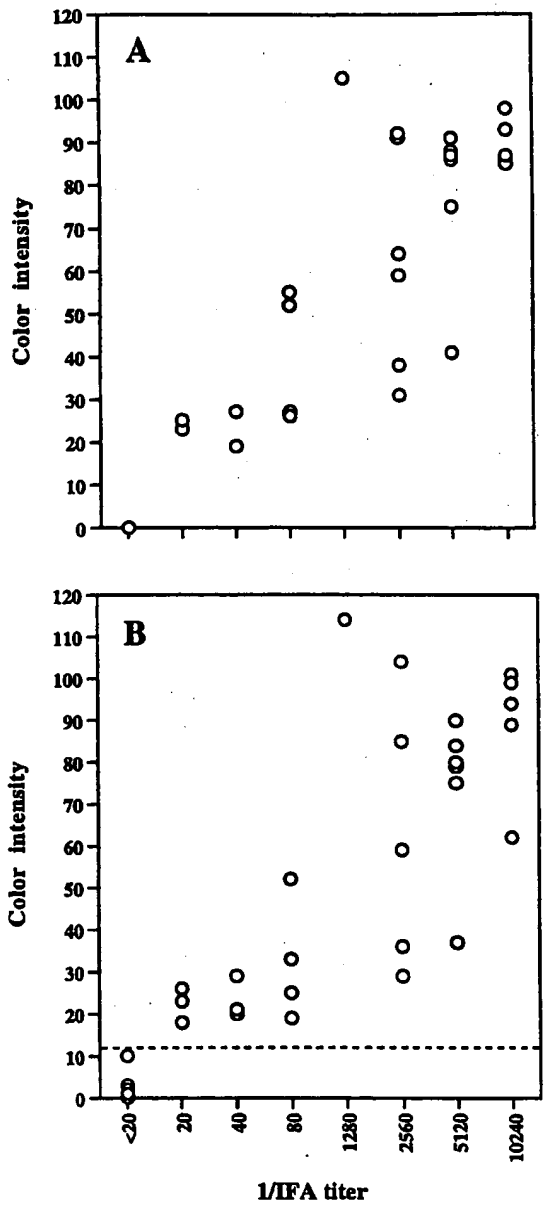


FIG. 9. Correlation between IFA titer (reciprocal dilutions) and color intensity of the dot immunoassay with purified *E. canis* antigen (A) and partially purified rP30 antigen (B). The color intensities of all dots in Fig. 8 were determined and plotted. Each circle represents one plasma specimen ($n = 42$). The correlation coefficients were 0.71 ($P < 0.001$) for graph A and 0.68 ($P < 0.001$) for graph B. The dashed line in graph B represents the cutoff value, which was determined from the highest color intensity in the immunoreaction with 13 negative plasma samples.

ples, 5 had color intensities of the purified organism antigen greater or lesser than those of the recombinant antigens. Additional major immunodominant proteins of *Ehrlichia* spp. are heat shock proteins (HSPs) (29, 33). Consequently, when anti-HSP antibody or antibody against protein antigen other than P30 is present in the plasma, whole organism antigens would give an immunoreaction stronger than that of the recombinant protein. On the contrary, when anti-P30 antibody is dominant in the plasma, the reaction with the recombinant protein would be stronger than that with the whole organism antigen. More importantly, the recombinant antigen-dot blot assay could clearly detect all of the 29 IFA-positive plasma samples. Furthermore, between native and recombinant antigens, no significant difference was observed in the correlation coefficient between IFA titers and the blot color intensity. Therefore, the rP30 antigen-immunodot blot assay offers advantages over the other serodiagnostic tests in general availability, ease of handling, and accuracy in the serodiagnosis of *E. canis* infection. Additionally, although it was not described in this paper, this *E. canis* recombinant antigen can be applied to enzyme-linked immunosorbent plate assays or other serodiagnostic assays as well.

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